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13. ABSTRACT (Maximum 200 Words)
It is widely presumed that environmental exposures play a role in the development of breast cancer, but few individual agents have been unequivocally identified as risk factors. Rather than seek out individual agents, we hypothesize that the cumulative effect of environmental exposures on an individual can be quantified through a blood-based assay, and further, that such a "biomarker" might distinguish breast cancer patients from age-matched controls. Preliminary evidence seems to support this hypothesis, and we have now begun to supplement this preliminary data in a manner that will allow us to determine how environmental exposures and predisposition interact with other know risk factors for breast cancer, such as family history, life history of hormonal exposure, and exposure to ionizing radiation. These biomarker data can then be added to a risk assessment procedure for breast cancer, and ultimately, might help identify the types of exposure specifically associated with cancer in the breast.

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Introduction

About 10% of incident breast cancer can be attributed to hereditary factors, and a further 15% can be accounted for by a complex mix of life history factors widely interpreted as representing endogenous hormone production. This leaves the great majority of breast cancer unaccounted for. It is assumed that environmental exposures are involved in breast carcinogenesis, and strong evidence for the effect of radiation has been presented. Total genotoxic exposure, especially the contributions of the countless chemicals in the environment, are impossible to calculate based on the agents themselves. Such exposure monitoring also does not integrate the individual response to genotoxic exposure that modulates its effect on processes such as carcinogenesis. We have proposed that direct monitoring of genetic effects at a surrogate locus in a easily available tissue can provide a biodosimter of environmental effects, and we have provided preliminary evidence that the blood-based GPA assay can provide this data. The current project is designed to expand our preliminary pool of retrospective data on 47 breast cancer patients to a more generalizable population of 200 patients, along with suitable controls for comparison. Acknowledged risk factors for breast cancer are also acquired to allow for the integration of the biomarker data into a more robust risk assessment paradigm for this disease.

Body

Risk factors for the development of breast cancer remain largely unknown, however, several clear elements have emerged: family history of breast cancer, metabolic factors related to hormone production, and exposure to X irradiation (1,2). It has been suggested that breast cancer incidence is also influenced by the accumulation of man-made chemicals in the environment. Two types of environmental chemicals have been implicated; those that mimic hormonal effects, known as "xenoestrogens", and those that mimic the DNA-damaging effects of X irradiation, or "genotoxicants". We hypothesize that breast cancer incidence should be a product of both the total cumulative exposure to genotoxic agents, including but not limited to X-rays, as modified by differences in individual response to this exposure as mediated by host factors, such as metabolic detoxification (or activation) and DNA repair capacity.

Although there is bound to be some element of tissue specificity for both genotoxic exposure and susceptibility to DNA damage, it is impractical to monitor somatic mutation in breast tissue itself. Blood, however, and its progenitor tissue bone marrow, are present throughout the body, and most xenobiotic exposures to the breast are likely to be transported to the breast tissue through the blood. The *GPA* assay is fast and inexpensive, utilizing flow technology to quickly quantify rare mutational events. However, due to its genetic basis, it can only be applied to individuals heterozygous for the MN blood group, which make up approximately 50% of the population. The *HPRT* assay is universally applicable, but requires cell culture and drug selection, making it more expensive and labor-intensive (3). Moreover, one class of HPRT mutants have been specifically identified as occurring via illegitimate V(D)J recombination (4), a mutagenic process that is characteristic of loss of double strand break DNA repair, such as in the cancer-prone syndrome ataxia telangiectasia (AT). The BRCA1 and BRCA 2 breast cancer

predisposition genes have also been implicated in this type of repair, so may also have a characteristic increase in these types of mutants (5).

This hypothesis was first applied to a mixed population of cancer patients, and these results, originally preliminary data for this proposal, have now been published (6). These data include significant contributions from cancers (breast, prostate, testicular) with acknowledged "hormonal" factors, suggesting that a dependence on genotoxic exposures is not mutually exclusive with an association with endocrine factors. We supplemented this data with analyses of local breast cancer patients, such that we had a population of 47 breast cancer patients analyzed prior to the submission of this grant. Analysis of the breast cancer patients alone confirmed that they followed the same trends as the mixed cancer population, and an odds ratio of 4.69 could be calculated for individuals with *GPA* mutation frequencies of 3×10^{-5} or higher.

Although the new HIPAA regulations caused us more delays, we have been recruiting patients into the original study design, and have completed *GPA* assays on 40 patients from Magee-Womens Hospital. Lymphocytes are frozen for *HPRT* analysis, and we have trained Ms. Christina Cerceo, a new technician now in her fifth month with us, to perform the assay (with seven successful control normal subjects). Although it is unlikely that we will complete our recruitment on the timeline of the original proposal, we will be applying for a no-cost extension to do so.

Amajor accomplishment of 2002 has been the integration of two mechanistic studies into the larger study. Through a DOD idead award (DAMD17-01-1-0605) we were able to develop a set of three PCR-based biomarkers specific to DNA double-strand break damage and repair. All three biomarkers analyze the products of illegitimate V(D)J recombination. The first molecularly quantititates the creation of an inverted 7 chromosome characteristic of the DNA repair deficient hereditary disease ataxia telangiectasia (AT) (7,8). This marker is caused by utilization of splicing sequences from two immunological genes on opposite arms of chromosome 7, the TCRγ gene at 7p14 and the TCRβ gene at 7q35. This marker can also be induced by environmental exposure (9), so it is similar to the somatic mutation assays in that it integrates both exposure and susceptibility. The second marker is based on a t(14;18) chromosomal translocation found in approximately 85% of follicular lymphomas by both cytogenetic and molecular analyses (10,11). This rearrangement deregulates expression of the *BCL-2* oncogene by juxtaposition into the *Ig* heavy chain locus by illegitimate V(D)J recombination (12). A sensitive PCR-based assay has been developed for the detection of this translocation in peripheral blood lymphocytes, and has demonstrated that the t(14;18) can be detected at low levels in almost all healthy individuals (13,14). Thus, this marker is produced by recombination of a normal splice signal in the *Ig* gene with a cryptic site in *BCL-2*. Finally, the third marker is based on a characteristic deletion of exons 2 and 3 in the X-linked *HPRT* gene that is also mediated by illegitimate V(D)J recombination. This deletion was first discovered in cord blood lymphocytes (15) and was subsequently found in adults (16). A quantitative PCR assay was developed to measure the frequency of this deletion in human peripheral blood cells (14,17), where its ontogeny appears to parallel the incidence of childhood leukemia and lymphoma (18). The generation of the marker requires the use of two cryptic V(D)J recombination signals in the *HPRT* gene, and is often applied to the molecular characterization of HPRT deficient clones from the *HPRT* mutation assay.

The second expansion of the original study design involves a collaboration with Dr. Jean Latimer of the Magee-Womens Research Institute. Dr. Latimer is an expert in another DNA repair system, nucleotide excision repair (NER), usually associated with repair of DNA damage caused by exposure to ultraviolet light (pyrimidine dimers, among other species) (19). She has also developed a reliable means of culturing normal breast epithelium and tumor (20, manuscript submitted to J Cell Biol). We have used these techniques to begin to determine whether loss or deficiency of NER could play a role in the higher somatic mutation frequencies we have documented in sporadic breast cancer patients. These studies were based on three observations: 1) NER activity modifies bodily response to a major class of environmental mutagens, bulky adducting long-chain and polyaromatic hydrocarbons, so is likely to be involved in most cancers that have a component of environmental exposure in their etiology; 2) since it requires the products of 25 genes, the NER pathway offers a large target for environmental mutagenesis; and, 3) early studies suggested that breast cancer patients (21) were deficient in NER.

Since the NER pathway is so complex, it is difficult to assay individual components, although a cell-free protein reconstruction system has been established (22). There are basically three methods for practically measuring NER, and all require actively proliferating cells: a) the unscheduled DNA synthesis (UDS) assay involving label incorporation during repair of damage caused by delivery of UV or a UV-mimetic chemical; b) the host cell reactivation assay (HCR), which involves repair of a transfected plasmid carrying a reporter gene with at least one NER-repairable lesion; or c) immunological monitoring of the reduction in UV associated DNA lesions, specifically pyrimidine dimers and/or 6-4 photoproducts, after delivery of UV or a UV-mimetic chemical. Of the three, the UDS assay is by far the most widely applied, having been used as a screen for genotoxic chemicals by the National Toxicology Program (23). The UDS assay was therefore our assay of choice for functionally assessing the entire NER pathway in the following studies.

Dr. Latimer and I had previously collaborated in a study using the *GPA* and UDS assays to characterize a newly diagnosed pediatric patients suffering from a Rothmund-Thomson syndrome, premature aging syndrome with elements of DNA repair deficiency (24). The results to date in our collaboration on breast cancer patients are summarized in **Figures 1**, **2** and **3** and **Table 1**. As tissue-specific controls, we analyzed a population of 22 normal breast epithelium tissue samples from reduction mammography patients (**Figure 1**, **Population A**). These data have been published (25, see Appendix). The results of UDS analysis of 17 stage I breast tumors are given in **Figure 1**, **Population C**. As a population, the breast tumors were significantly lower in NER capacity than the breast reduction samples, exhibiting an average of 47% normal activity ($P = 0.0002$). None of the breast tumor cultures exhibited an NER capacity equal to the average of the reduction mammoplasty samples, and half of the tumors had NER capacities lower than that of the lowest breast reduction. As shown in **Table 1**, if 70% of normal NER activity is established as a cut-off for these data the UDS assay is able to discriminate normal and tumor samples with a sensitivity of 88%, a specificity of 78% and a significant odds ratio (OR) of **27.0**. This result clearly establishes that loss or deficiency of NER is associated with breast cancer development. These data have been finalized and are the subject of a manuscript now under consideration for publication in the *Journal of the National Cancer Institute* (see Appendix). These data are consistent with our somatic mutation results, but appear to be a more powerful biomarker for breast cancer susceptibility.

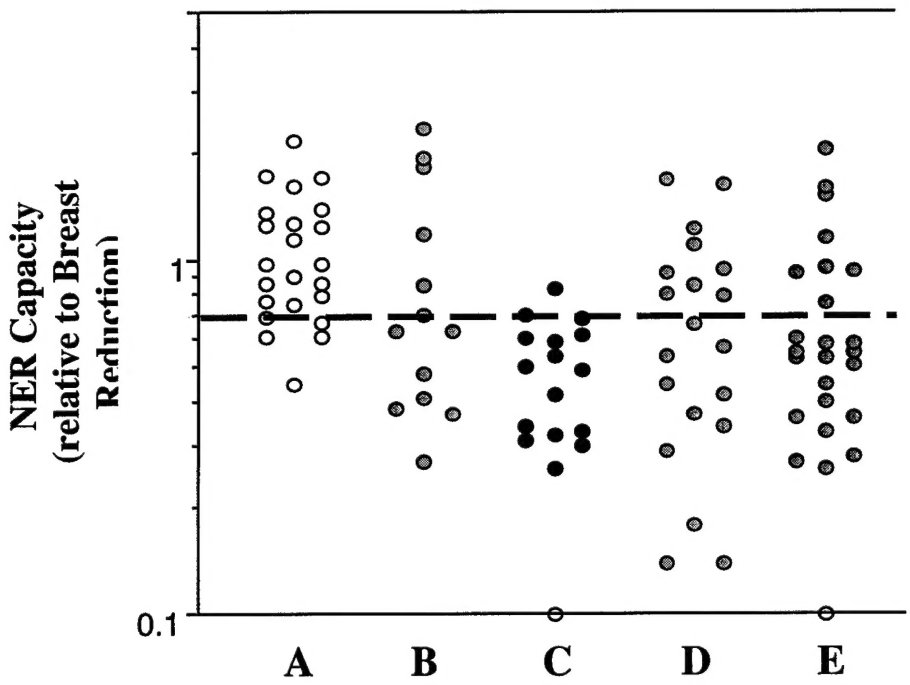

Figure 1. NER capacity of primary cultures from normal breast epithelium and early stage tumors. NER capacity was assessed with the UDS assay on primary explants and expressed relative to the average activity of tissue from disease-free women undergoing breast reduction mammoplasties. **A**) normal breast epithelium (including one contralateral sample from a breast cancer patient (N = 23); **B**) "non-tumor adjacent" (NTA) tissue from 13 stage I breast cancer patients; **C**) tumor tissue from 17 stage I breast cancer patients; **D**) NTA tissue from 20 stage II breast cancer patients; **E**) tumor tissue from 25 stage II breast cancer patients. The dotted line represents 0.70 X the mean of the breast reduction population, the cut-off established in **Table 1**.

Population	Total Assayed	NER Capacities[1] < 0.70	> 0.70	OR[2] (95% CI[3])
breast cancer patients (stage 1 only)	17	15	2	**27.0** (14.4-50.8)
breast cancer patients (stages 1 + 2)	42	32	10	**11.5** (7.25-18.3)
disease-free controls	23	5	18	

Table 1. Increased proportion of tissue samples with low NER capacities in breast cancer patients vs. controls. [1]relative to average of breast reduction samples, [2]odds ratio, [3]confidence interval

In 12 cases out of the above 17 stage I tumors, we were also able to obtain, successfully culture and analyze the NER capacity of a sample of histologically non-tumor epithelial tissue from the area adjacent to the excised tumor (**Figure 1, Population B**) (we also analyzed one "NTA" sample from an early stage tumor that failed to grow in culture). Our expectation was that this normal tissue would exhibit normal levels of NER, establishing that NER deficiency arises as a somatic event during oncogenesis. Instead, these stage I NTA data appear to span the full range of both reduction and tumor results; averaging 89% of normal activity; they are nevertheless significantly lower in NER capacity as a group than the breast reductions ($P <$

0.0001), and significantly higher than the tumor samples ($P = 0.01$). Breaking the paired sample sets into populations based on their NER capacity (using the cut-off established in **Table 1**), reveals two distinct populations of breast cancer patient, a minority that exhibit normal NER capacity in their breast epithelium and low NER capacity in their tumor (**Figure 2, Panel A**, 3/12, 25%), and a majority that exhibit reduced NER capacity in both normal and tumor tissue (**Figure 2, Panel B**, 9/12, 75%). If the NTA samples do indeed represent the normal level of NER in these cancer patients, these data suggest that the majority of breast cancer patients constitutively manifest low levels of NER. This observation would be applicable as a screening tool for breast cancer risk, if it could be performed economically.

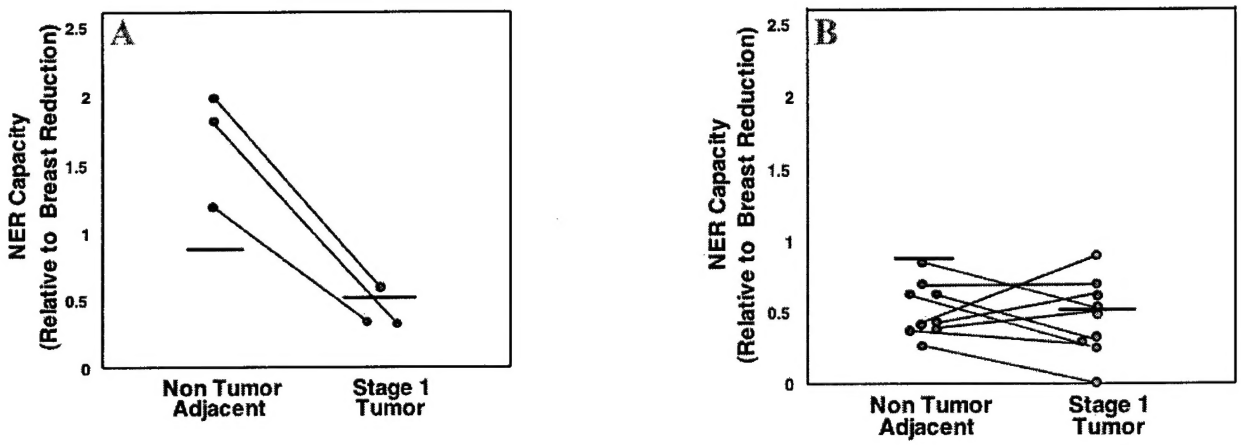

Figure 2. Matched sets (joined by lines) of stage I breast tumor and NTA samples from **Figure 1** broken into those patients whose NTA sample exhibited A) greater than average, or B) less than average NER capacity relative to normal controls. The three tumors in panel A had much reduced NER capacity relative to their NTA sample, presumably due to a somatic event such as mutation. The nine tumors in panel B changed relatively little in terms of NER activity, since these patients had less than average repair capacity in their normal tissue to begin with (horizontal lines indicate the average NER capacity of the population from which these samples were derived).

We are now in the process of completing our analysis of stage II breast tumor samples. UDS results from 25 such samples are given in **Figure 1, Population E**; not all of these data have been finalized, however, as some samples have not been confirmed histologically from the block made from the sample put into culture (also, some of the samples with high NER values were derived form patients with multifocal disease, and may not be true stage II tumors). Nevertheless, these preliminary stage II NER data share many characteristics with the stage 1 results. They exhibit a mean NER capacity of 0.69 ± 0.09 relative to the mean of the normal controls, significantly lower than the breast reduction controls ($P = 0.004$), but not significantly different from the NER capacity of the stage I samples ($P = 0.07$). If these data are added to the stage I results and similarly evaluated, the UDS assay with a cut-off of 70% normal activity can discriminate normal and tumor samples with a sensitivity of 76% and a significant OR of 11.5 (**Table 1**). These data are a significant improvement over the GPA assay results, and suggest that NER measurements would be a better risk assessment tool for breast cancer.

We have also performed the UDS assay on NTA samples from 24 of the 25 stage II breast cancer patients (**Figure 1, Population D**), and analysis of these slides is ongoing. Preliminary results on 20 such samples indicates that the NER capacities of these samples are almost identical to those of the stage II tumors at 0.70 ± 0.10 of breast reduction normal (and not significantly different, $P = 0.91$), and are still significantly lower than normal ($P = 0.004$). If the same criteria are applied to the UDS data on these 20 matched sets of tumor and NTA tissue, 13 (65%) are NER deficient in both samples, 3 (15%) show a dramatic loss of NER only in the tumor, and 4 (20%) define a new category where both samples are similar and in the normal range (0.70 and above), suggesting that modulation of the NER pathway is not involved in their etiology. Overall, these preliminary data on stage II breast cancer patients support our earlier conclusion: the majority of breast cancer patients constitutively manifest low levels of NER which can be detected in breast tissue samples with the UDS assay.

Although some clinical uses could be proposed for the results of our UDS analysis in breast tissue, it is unlikely that we will be able to screen populations with this technique. Instead, we would like to establish that the NER deficiency observed in the normal breast samples from breast cancer patients (the NTA samples) can be detected in a peripheral tissue, such as blood. The UDS assay can be performed diagnostically on peripheral blood lymphocytes (PBL) (26), and we recently published a large survey of normal individuals analyzed this way (N = 33) (25, see Appendix). Unfortunately, that study also established tissue-specific differences in NER capacity between fibroblasts, lymphocytes and epithelial cells, such that coordinate regulation of the three cell types cannot be assumed. A preliminary analysis of nine blood samples from newly diagnosed breast cancer patients, however, suggests that a blood-based assay of NER deficiency is feasible (**Figure 3**). Based on our stage I tumor results, we would expect ~seven of these patients to exhibit NER deficiency averaging 50% of normal activity. In fact, our lymphocyte population averaged only 0.23 ± 0.09 NER capacity relative to the mean of our control population, with the highest value at the cut-off established in the breast tissue/tumor study, 0.70. The cancer patients were significantly lower in lymphocyte NER capacity than controls ($P = 0.005$). These samples were not from the same patients as the tumor samples, however, so further comparisons cannot be made.

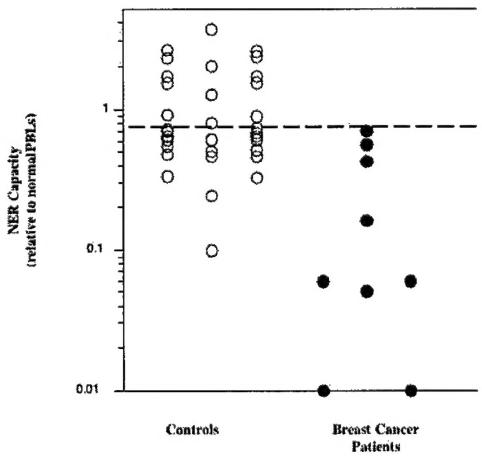

Figure 3. NER capacity of PBLs from disease-free controls (N = 33) and newly diagnosed (pre-therapy) breast cancer patients. NER capacity was assessed with the UDS assay.

Key Research Accomplishments

- We have begun patient recruitment, sampling and assay application.

- We have incorporated a set of three PCR-based biomarkers of DNA double-strand break damage and repair into the study.

- We have incorporated a functional analysis of NER capacity into the study, with very promising preliminary results, including publication of the normal controls, and pending publication of the stage I tumor data.

Reportable Outcomes

- Our preliminary studies on non-diseased breast epithelial tissue and PBL samples has been published:

Latimer JJ, Nazir T, Flowers LC, Forlenza MJ, Beaudry-Rodgers K, Kelly CM, Conte JA, Shestak K, Kanbour-Shakir A, Grant SG. (2003) Unique tissue-specific level of DNA nucleotide excision repair in primary human mammary epithelial cultures. Exp Cell Res 291: 111-121.

- Our initial studies on stage I breast epithelial tissue samples has been presented at two national meetings, and has been submitted for publication:

Latimer JJ, Kelly CM, Patterson JA, Vogel V, Grant SG, Johnson J. (2002) Molecular etiology of DNA repair deficiency in novel non-tumor adjacent and tumor cell lines. Presented at the 2002 *Department of Defense Breast Cancer Research Program Era of Hope Meeting*, Orlando, Florida.

Kelly CM, Johnson JM, Wenger SL, Vogel V, Kelley J, Johnson R, Amortequi A, Mock L, Grant SG, Latimer JJ. (2003) Analysis of functional DNA repair in primary cultures of the non-tumor adjacent breast identifies two classes of breast cancer patient. Presented at the 2003 meeting of the *American Association for Cancer Research*, Washington, DC. Proc Am Assoc Cancer Res 44: 974-975.

Latimer JJ, Kelly CM, Kelley J, Johnson R, Kanbour-Shakir A, Vogel VG, Grant SG. Intrinsic DNA nucleotide excision repair deficiency in sporadic breast cancer. J Natl Cancer Inst (submitted).

- In association with a local study to screen patients at high risk for breast cancer, we have submitted an analysis of somatic mutation and NER capacity in BRCA1 carriers:

Latimer JJ, Rubinstein W, Cerceo CM, Grant SG. Normal levels of DNA nucleotide excision repair in breast tissue and blood lymphocytes of a *BRCA1* mutation carrier. Clin Breast Cancer (submitted).

Conclusions

In addition to finally getting the study underway this year, we have also begun the follow-up studies that will attempt to establish the mechanism or mechanisms behind our initial observations. To our surprize, it appears that genetic factors may actually play a larger role than exposure. This is fortunate, since patients carry their genome intact and available for analysis throughout their lifetime, whereas evidence of environmental exposure is often subtle to begin with and fades with time. In the coming year, we plan to publish our inital somatic mutation results, our UDS results on stage I NTA and stage II patients, and to design studies to confirm and extend our demonstration of NER deficiency in breast tumors and normal tissue from breast cancer patients.

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Appendices

Latimer JJ, Nazir T, Flowers LC, Forlenza MJ, Beaudry-Rodgers K, Kelly CM, Conte JA, Shestak K, Kanbour-Shakir A, Grant SG. (2003) Unique tissue-specific level of DNA nucleotide excision repair in primary human mammary epithelial cultures. Exp Cell Res 291: 111-121.

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Unique tissue-specific level of DNA nucleotide excision repair in primary human mammary epithelial cultures

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Abstract

DNA repair is essential for the maintenance of genomic integrity and stability. Nucleotide excision repair (NER) is a major pathway responsible for remediation of damage caused by UV light, bulky adducts, and cross-linking agents. We now show that NER capacity is differentially expressed in human tissues. We established primary cultures of peripheral blood lymphocytes (PBLs: N = 33) and foreskin fibroblasts (FF: N = 6), as well as adult breast tissue (N = 22) using a unique culture system, and measured their NER capacity using the unscheduled DNA synthesis (UDS) functional assay. Relative to FF, primary cultures of breast cells exhibited only 24.6 ± 2.1% of NER capacity and PBLs only 8.9 ± 1.2%. Cells from the breast therefore have a unique and distinctive DNA repair capacity. The NER capacities of all three cell types had similar coefficients of variation in the range of 10%–15%, which should be taken into account when running controls for this contextual assay. Unlike previous studies and speculation in the field, we found that NER was not affected by cell morphology, donor age, or proliferation as measured by the S phase index. While the NER capacity of the transformed lymphoblastoid cell line TK6 was within the range of our PBL samples, the breast tumor-derived MDA MB-231 cell line was four-fold higher than normal breast tissue. These studies show that analysis of baseline DNA repair in normal human cell types is critical as a basis for evaluation of the effects of "mutator" genes as etiological factors in the development of cancer.

Keywords: Human mammary epithelial cells; HMECs; Nucleotide excision repair; NER; DNA repair; Tissue specificity; Unscheduled DNA synthesis assay; UDS

Introduction

Living organisms are subjected to a constant barrage of DNA damage caused by exposure to a wide range of exogenous chemical and physical agents, as well as endogenous metabolic processes. Unrepaired DNA damage may lead to the accumulation of somatic mutation and the eventual development of neoplastic transformation.

Three types of genes are known to be involved in the etiology of most cancers: oncogenes [1–3], tumor suppressor genes [4,5], and the subset of tumor suppressor genes known as "mutator" genes, usually involved in DNA repair [6–8]. Mutations in DNA repair genes compromise the long-term ability of a cell to correct genotoxic damage. DNA repair deficiencies can result in an accelerated rate of cellular mutation, potentially serving as the step that confers genomic in-

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stability. There are multiple pathways of DNA repair in man, including double-strand break repair, base excision repair, base mismatch repair, and nucleotide excision repair [9].

Long patch, or nucleotide excision repair (NER), is the primary process by which cyclobutane pyrimidine dimers, 6-4 photoproducts, and DNA cross-links are removed from the DNA [10,11]. $UV_{254\ nm}$ light, as well as so-called UV mimetic drugs, induce DNA lesions that are corrected by this pathway. Damage lesions caused by genotoxic chemotherapy agents that act as inter- and intrastrand cross-linkers, such as cisplatin [12], covalently bind to DNA creating "bulky" adducts. Other bulky adducts are caused by agents such as *N*-acetoxyaminoacetylfluorene (AAAF) and melphalan [13,14] and alkylating agents, such as cyclophosphamide [15,16] are also presumably remediated by this pathway. NER is a complicated process requiring the protein products of 20–30 genes [17]. NER involves the recognition of a damage lesion causing distortion of the DNA helix, incisions flanking the lesion on the damaged strand, excision of 27–29 bases including the damage lesion, and replication and ligation to replace the excised information and seal the strand breaks at each end of the newly synthesized region [18–21]. This pathway can also be called into play for other types of DNA damage lesions that have not been corrected by base excision and other single-stranded DNA repair mechanisms [22,23]. In effect, the NER pathway provides redundancy for these other repair systems should they be overwhelmed by a genotoxic exposure [10].

NER of the overall genome can be measured quantitatively using the unscheduled DNA synthesis assay (UDS). The UDS assay involves the measurement of labeled base incorporation into the DNA after in vitro exposure to UV light or certain chemicals. The UDS assay is a cell autonomous, functional assay, in that it allows one to look at the complex process of NER as a whole, at least as it is expressed in a particular cell type [24–26]. As applied in our laboratory, this assay predominantly quantifies the repair of UV-induced DNA 6-4 photoproducts, and elements of both the "global genomic" as well as the "transcription coupled" components of NER contribute to the results [10,26].

The autoradiographic UDS assay requires the analysis of living cells. It has previously been applied primarily to skin fibroblasts and peripheral blood lymphocytes (PBLs) for diagnosis of xeroderma pigmentosum (XP) and other DNA repair diseases impacting specifically on the NER pathway. Classical NER deficiency disorders are characterized by UV sensitivity manifesting mainly in the skin and cornea. In diagnostic studies, a single "normal" sample of the same tissue type is used as a control. Alternatively, foreskin fibroblasts (FF) have historically been used as positive standards in UDS studies, because these cells manifest a consistently high level of repair. Mixtures of FF from several babies have sometimes been used to attempt to account for possible interindividual differences. PBLs are among the most accessible nucleated cell types in the body, and have therefore been used in studies to determine the DNA repair capacity of cancer patients and their relatives. UDS analysis of PBLs and skin fibroblasts have seldom been reported concurrently, however, and never in a form that would allow the two to be placed into the same context.

Studies involving functional assays in general, and specifically functional assays of DNA repair capacity, have been hampered by a technical lack of ability to perform primary explant culture on all cell types. The one notable exception is that of rat hepatocyte primary cultures, which have been used extensively in UDS assays for evaluation of the carcinogenic potential of chemicals [27,28]. Although repair assays can be performed on established, transformed cell lines, the generation of cell lines from normal adult tissue has proven to be a technical challenge. In addition, during the process of passaging, established cell lines undergo clonal evolution that may alter or extinguish many of the original characteristics of the cells, including their intrinsic repair capacity [29,30].

Until recently, cell culture techniques have not existed to support primary culture explants of most human tissues. These tissues require attachment to a substratum of some sort of extracellular matrix (ECM). In particular, lineages that involve epithelial cells have proven to be extremely difficult to culture, because these cells normally rest on a complex, biologically reactive basement membrane produced in vivo by the cell type residing beneath the basal surface of the epithelial cells, the myoepithelial cells [31,32]. We have developed a novel system for establishing primary cultures of breast epithelial cells and stromal fibroblasts [33]. Because most breast tumors arise from epithelial cells, the evaluation of their baseline mutator gene function, i.e., DNA repair capacity, is extremely important, and is now possible in primary epithelial cultures.

It has long been presumed that DNA repair is so essential to the maintenance of genomic integrity that it is constitutively expressed in all cells within an individual. A natural extension of this assumption is that DNA repair capacity is equivalent in all cell-types within an individual. However, the possibility that NER can be regulated is illustrated by the findings that NER is present in different levels in various tissues and cell types in mammalian development. In a previous study of the excision repair capacity of the four distinct extraembryonic lineages that comprise the extraembryonic yolk sac, as well as five cell types derived from the fetus, we have shown that NER in the mouse is lineage-specific during embryogenesis [34]. In the present study, we expand this observation from mouse embryonic tissues to a small sampling of human tissues, including, for the first time, breast epithelial cells.

Materials and methods

Tissue procurement and establishment of cultures

Breast reduction mammoplasty tissues were obtained from patients at Magee-Womens Hospital under Magee-Womens Hospital/University of Pittsburgh IRB # MWH-94-108. A neighboring piece of mammoplasty tissue (from

the same 0.25 cm^2 sample) to that placed into primary culture was fixed and embedded in paraffin. Sections were examined by a pathologist to verify the histological normality of the tissue.

Peripheral blood lymphocytes (PBLs) were obtained with consent from normal healthy control subjects working at Magee-Womens Hospital, Magee-Womens Research Institute or students at the University of Pittsburgh. Foreskin fibroblast (FF) tissue was obtained as discarded tissue from newborn infants after circumcision.

FF were converted into primary explants as described in Latimer et al. [34]. Briefly, cells were grown in MEM containing 10% fetal calf serum on uncoated chamber slides (Nalge Nunc International, Naperville, IL). These cells were passaged to promote homogeneity and grown continuously in culture for up to 12 passages. These cultures show constant levels of DNA repair until passage 13 and senesce after approximately 20 [35]. For UDS experiments, FF were utilized between passages 7 and 10. Four different preparations of FF were used in this study: one consisted of a pool of three perinatal circumcisions and the remaining three were made from individual circumcisions.

PBLs were obtained from normal healthy male and female controls ages 20–50. Lymphocytes were purified using the ficoll gradient method [36] and placed onto a diluted form of Matrigel (BD Biosciences, Bedford, MA) in RPMI medium supplemented with 15% fetal calf serum. It was discovered that PBLs adhered to Matrigel-coated chamber slides, so the autoradiographic UDS assay could be performed on these adherent cells [37]. Lymphocytes were cultured 5–7 days before performance of the UDS assay. Nineteen out of the 33 samples presented here have been previously published as controls in a study on the effects of stress on NER capacity [37].

Breast reduction mammoplasty tissue was rinsed, processed, and placed into primary cultures within 5 h of surgery. Tissue was mechanically disaggregated and placed on diluted (1:1) Matrigel in a novel tissue culture medium called MWRIα according to a method developed in our laboratory [33]. It has been well established that Matrigel provides an optimal commercially available surface for attachment of epithelial cells [38]. The extracellular matrix (ECM) components of matrigel are apparently close enough to the natural basement membrane for mammary luminal epithelial to adhere and retain a rounded morphology [39]. We therefore used matrigel for both establishment of breast cell cultures and to allow the normally nonadherent PBLs to attach to the solid-surface of the glass slides used for the UDS assay. Primary breast cultures were grown for 7–10 days, imaged using a digital Hammamatsu video camera, and then analyzed for NER capacity using the UDS assay. UDS experiments were performed when the epithelial cells were present as mammospheres (clusters of epithelial cells numbering from 40 to 150 cells [40]). Stromal fibroblasts were also readily distinguishable in these cultures.

TK6 lymphoblastoid cells and MDA MB-231 stage IV breast tumor cells were purchased from the American Type Culture Collection (Rockville, MD).

Unscheduled DNA synthesis assay

NER was measured using autoradiography of unscheduled DNA synthesis (UDS) [25]. After a total of 7 days in culture, without passaging, cultures were irradiated with UV light at 254 nm at a mean fluence of 1.2 J/m^2 for 12 s in the absence of culture medium, for a total dose of 14 J/m^2. Primary cultures had not reached confluence and were still actively growing at the time the UDS assay was performed. Control established cell lines were plated subconfluently 1–2 days before the UDS assay to ensure that they also were not in a quiescent state brought on by confluence. Careful UV dosimetry was performed using a UV delivery system specifically designed for this assay [41]. This machine contains three UV germicidal bulbs placed at a distance of 3 feet (91.4 cm) from an electric turntable where the chamber slides are placed. A 6-in diameter photographic shutter opens under electronic control to deliver a precisely timed dose of UV light. UV bulbs were warmed up at least 1 h before UDS and UV output was checked before each experiment using a Spectroline DM-254X UV meter.

Each sample was represented by at least two chamber slides. One chamber of each two-chamber slide was shielded from the UV dose to be used as an unirradiated control sample. After UV exposure, all cultures were incubated in medium supplemented with 10 μCi ml [^{3}H]methylthymidine (~80 Ci $mmol^{-1}$) (PerkinElmer Life Sciences, Boston, MA) for 2 h at 37 °C. Labeling medium was then replaced with unlabeled chasing medium containing 10^{-3} M nonradioactive thymidine (Sigma, St. Louis, MO) and incubated for a further 2 h to clear radioactive label from the intracellular nucleotide pools. After incubation in the post-labeling medium, cells were fixed in 1X SSC, 33% acetic acid in ethanol, followed by 70% ethanol and finally rinsed in 4% perchloric acid overnight at 4°C. All slides were dried and subsequently dipped in photographic emulsion (Kodak type NTB2) and exposed for 10 to 14 days in complete darkness at 4 °C.

The length of exposure of emulsion was determined in each experiment by preparing "tester" slides. These are extra slides of the positive controls, for these experiments consisting of two slides each of FF and of the established breast cancer cell line MDA MB-231 [42]. These slides were dipped in photographic emulsion, dried, and packaged separately from the rest of the experiment in a sealed slide box. After 10 days these tester slides were developed and grain counting was performed. If the nuclei over the foreskin fibroblasts averaged 50 or more grains per nucleus, then the rest of the experimental slides were developed (including additional FF and MDA MB-231 slides). If the grain count was below this level, the remaining slides were left to expose 1–3 days longer before being developed. The tester slides were only used to determine when the exposure

time was optimal, because exposure time can vary depending primarily on the age of the radiolabel and the emulsion.

Grain counting analysis

After photographic development of the emulsion on the slides, the nuclei were stained with Giemsa, then examined at 1000X magnification on a Zeiss Axioskop under oil emersion for grains located immediately over the nuclei of non-S phase cells (S phase cells were distinguished by their high grain counts, at least 10-fold higher than non-S phase, and by a clustered pattern of grains). Local background grain counts were determined in each microscopic field, over an area the same size as a representative nucleus, and this total was subtracted from the grain count of each nucleus in that field. The average number of grains per nucleus were quantified for each side of the chamber slide, both unirradiated and irradiated. The final NER value for each slide was calculated by subtracting the unirradiated mean grains per nucleus from the irradiated mean grains per nucleus, after the initial subtraction of local background in each field. NER was ultimately expressed as a percentage of the activity of concurrently analyzed FF. An average of 4.6 FF slides were scored per experiment, with an average of 145 nuclei per slide, for a total of almost 700 nuclei for each FF sample, with an average of 42.6 grains/nucleus. An average of 2.9 slides were scored for each PBL sample, with an average of 205 nuclei/slide. Thus, almost 600 nuclei were counted for each PBL sample, with an average of 6.6 grains/nucleus. Finally, an average of 4.9 slides were evaluated for each breast reduction sample, with an average of 144 nuclei per slide. An average of 700 nuclei were therefore scored per breast reduction sample, with an average of 14 grains/nucleus.

Statistical analysis

To ensure accuracy and guard against transcription errors, raw grain counts from the UDS assay were processed independently in duplicate, once using StatView (version 5.0.1, SAS Institute, Inc., Cary, NC), and once using the Data Analysis Toolpack of the Excel 2001 spreadsheet program (Microsoft Corp., Redmond, WA). The final count from slides of the same cell type within the same experiment and developed the same day were averaged together and expressed as a percentage of concurrently analyzed FF, or, in the case of FF, a percentage of concurrently analyzed MDA MB-231. NER values for cell lines and FF controls were averaged over all experiments. Comparisons between different cell lines and cell types were performed using both the parametric two-tailed t test and the nonparametric Mann-Whitney U test, with significance determined at alpha < 0.05. In all cases, the P value reported is the higher of the two, which was always generated by the nonparametric test. Comparison of UDS values for cells with epithelial and fibroblastic morphologies from the same samples was performed using a paired t test at alpha < 0.05. The possible effects of donor age, S phase index, and cell type on NER capacity were evaluated using linear regression at the same level of significance.

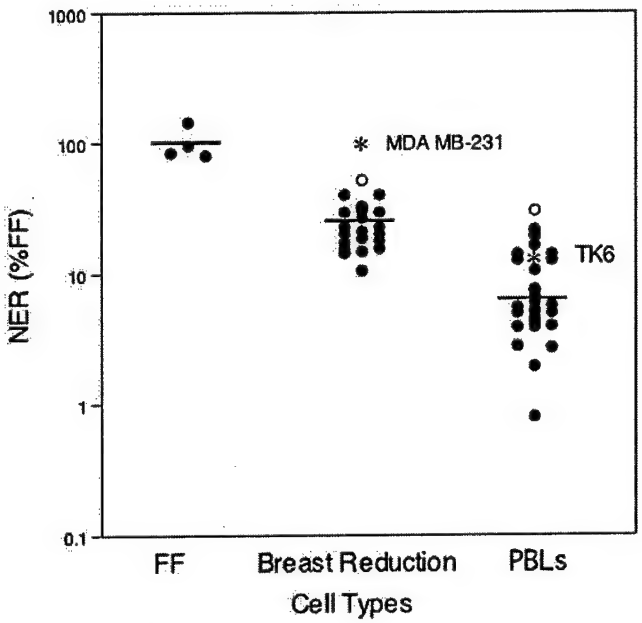

Fig. 1. NER capacity of different cell types. NER capacity of FF (arbitrarily set at 100%, $N = 6$), primary cultures of breast cells (24.6 ± 2.1%, $N = 22$), and PBLs (8.9 ± 1.2%, $N = 33$) from normal healthy newborns, women, and adults, respectively. Outliers are represented by open circles (O). The NER capacity of two transformed cell lines derived from the same tissue of origin as the primary cultures are indicated by asterisks in the breast reduction and PBL columns. The mean of each data set is shown by the horizontal line. NER capacity is expressed using a log scale.

Results

Tissue specificity of NER

Because UDS is a relative measure of DNA repair capacity, primary cultures of human FF were included in each experiment to serve as a standard of comparison. Subjects used for generation of FF cultures were all within the first week of life. PBL donors were recruited from laboratory and hospital workers and ranged from 20 to 50 years of age ($N = 33$). Breast reduction patients who provided normal tissue for culture ranged in age from 20 to 70 years of age ($N = 22$). The established breast cancer pleural effusion-derived cell line MDA MB-231 [42] was also included as a second, supplementary control in all experiments.

Histological evaluations were performed on breast reduction tissue adjacent to that placed into culture, on a block provided from the same 0.25 cm^2 sample that was fixed and processed in paraffin. Normal breast tissue can display an array of nonmalignant histologies that include fibrocystic changes, hyperplasia (overgrowth), and calcification. These changes are typical of normal breast tissue [43], and none of the samples selected for UDS analysis contained changes that were outside of this range of normal

As shown in Fig. 1, our results with the UDS assay revealed distinct DNA repair capacities for all three types of cultures analyzed. Although defined as 100% repair capacity in each experiment, comparison of the FF with MDA MB-231 allowed us to calculate the variability exhibited by our four lots of FF; one of which was pooled from tissue from three infants and three of which were derived from single individuals. Thus, our FF samples showed a coefficient of variation (COV) of 15%. Previous studies, because they have each used a single unique culture of FF as their normal "standard," have essentially assumed that there was no variability in FF NER capacity. PBLs, another cell type that has been often analyzed with the UDS assay, exhibited only 8.9 ± 1.2% (mean ± standard error) of FF NER capacity, demonstrating that there is a greater than 10-fold range in NER capacities among normal human cell types. This level of DNA repair was significantly lower than that of concurrently analyzed FF ($P = 0.001$), with a similar interindividual variability (a COV of 14%). Because we did not mitogen stimulate our PBL cultures, these results are consistent with previous studies suggesting unstimulated PBLs are "deficient" in NER [44,45].

Primary breast cell cultures exhibited an intermediate NER capacity of 24.6 ± 2.1% of FF levels, distinct and significantly different from both FF ($P = 0.002$) and from PBLs ($P < 0.001$). The interindividual variability of the breast reduction samples was again similar to the other two cell types (COV = 9%). Interestingly, both the PBL and breast reduction results contained one point that was significantly higher than the rest of the population (30% of FF for the PBL sample, 50% of FF for the breast reduction sample). Both of these values are greater than three standard deviations higher than the mean of the rest of their populations, suggesting that they might be considered as "outliers." Removal of these outliers did not affect the level of significance of the difference in NER capacity between these tissue types, nor did the outliers seem to affect the other statistical comparisons reported in the following sections.

Of the three types of cell samples tested, only the PBLs were available from donors of both sexes. Twenty-four of our PBL samples came from women, with an average NER capacity of 9.1 ± 1.6% of FF (8.2 ± 1.4% without the outlier). The eight male PBL samples were not significantly different in NER capacity from the female, averaging 8.7 ± 1.3% of FF ($P = 0.86$). Again, sex has not always been expressly matched in UDS studies that used adult controls rather than foreskin fibroblasts; this small comparison suggests that such matching might be unnecessary, at least for comparison of lymphocyte samples.

Analysis of breast cells by morphology

Because there was no attempt at cell-type selection or enrichment other than culture conditions, the primary breast tissue cultures contained cells of two distinct morphologies: epithelial cells which clustered together in three-dimensional organotypic structures called "mammospheres" [40], and more fibroblastic cells that grew as an underlying monolayer, and which might represent the stromal compo-

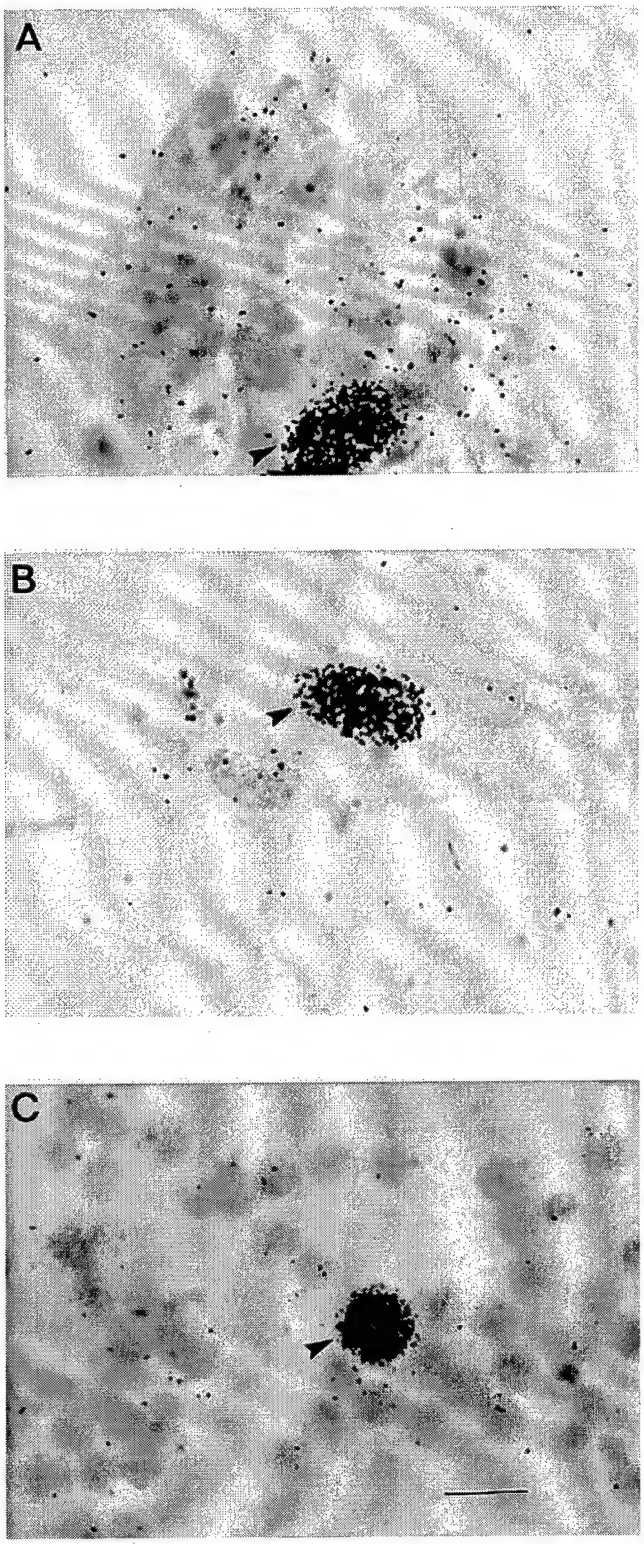

Fig. 2. Normal breast cells from reduction mammoplasty (A, B) and PBLs (C) after the UDS assay (1000X photomicrographs, bar = 100 microns). The field in (A) contains a cluster of mammary epithelial cells (mammosphere), while the field in (B) contains fibroblastic cells. Cells with intensely dark nuclei, such as those indicated by arrows, are in S-phase.

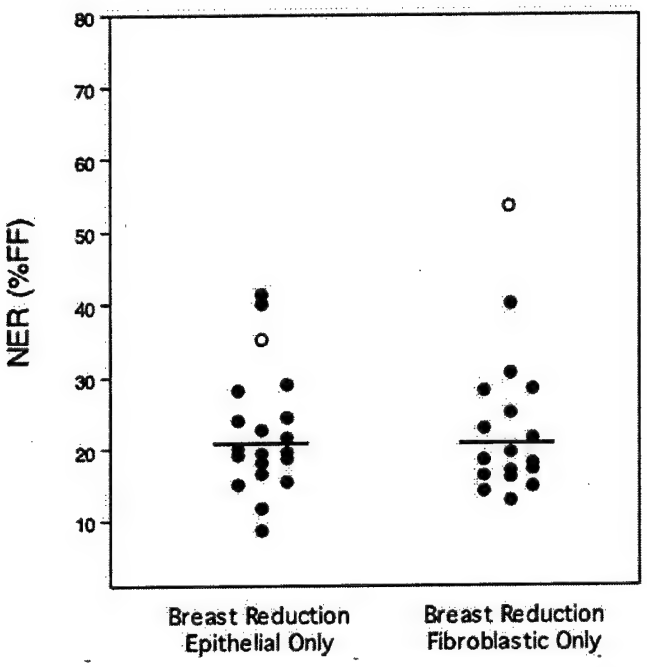

Fig. 3. Stratification of breast cell UDS results by cellular phenotype. No significant difference was observed in the NER capacities of cells exhibiting an epithelial vs a fibroblastic morphology. The outlier identified in Fig. 1 is represented by open circles (O). The mean of each data set is shown by the horizontal line.

nent of the breast (Fig. 2). Because we used the autoradiographic version of the UDS assay, we were able to analyze the NER capacity of both types of cells in our experiments. Epithelial cells were easily identified morphologically as rounded cells that stain with epithelial specific antigen (ESA), whereas the fibroblastic cells do not (data not shown). Tissue lymphocytes are not generally present in our normal breast tissue cultures unless some type of biopsy has been performed on the breast previous to surgery. This was not true of any of the breast reduction cases analyzed in this study. In any case, PBLs have a round cell morphology that is almost entirely nuclear, are present as singleton cells, and are much smaller than breast cells (Fig. 2).

Three breast cell samples yielded assayable cells that were entirely epithelial in morphology and one sample was entirely fibroblastic in morphology, but the average fraction of epithelial-like cells for all samples was 54%. Interestingly, separate quantification of NER in the epithelial and fibroblastic cells from the normal breast revealed no statistically significant difference between the two cell types, either on a population basis ($P = 0.53$) or within individuals ($P = 0.09$; 0.17 without the outlier) (Fig. 3). The NER level exhibited by the epithelial cells from these samples averaged 22.5 ± 1.9% of FF, whereas the fibroblastic cells from the same cultures exhibited an average NER capacity of 25.7 ± 2.7%. Both cell-type subsets from the pooled outlier sample had high NER capacities (35% of FF for the epithelial cells), but the effect was greatest in the fibroblastic cells, 53.4% of FF, which was an outlying value itself. Removal of the outlier reduced the NER capacity of the breast fibroblastic cells to 24.1 ± 2.3% of FF. The NER capacities of the epithelial and fibroblastic cells from the same individuals were highly correlated ($P = 0.003$), suggesting that genetic factors modify DNA repair activity similarly in both cell types.

Effects of age and proliferative index

Two factors have previously been proposed to affect DNA repair capacity: age of donor [46–48], and the mitotic activity of the cell sample [20,49]. It has been observed that uninduced human somatic mutation increases with age when measured in a number of different ways [50,51]. It has been suggested that this is consistent with a generalized loss of DNA repair capacity with age [52], and some data on NER in fibroblasts and PBLs seem to support this hypothesis [53,54]. Analyses of our NER data for a possible effect of age does not support such a hypothesis, however (Fig. 4). Indeed, a significant positive association between NER capacity and age was observed for the PBL data ($P = 0.01$). This result should be viewed with caution for two reasons: the relative paucity of samples from older individuals (only four would be considered postmenopausal), and the potential effect of the "outlier" mentioned earlier, who was one of

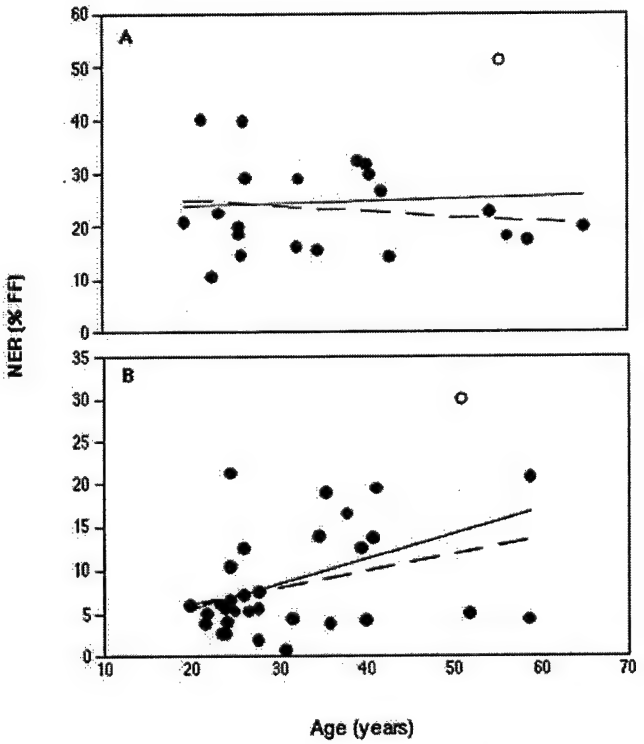

Fig. 4. Age regression graph of NER capacity for (A) breast cells, and (B) PBLs. The aberrantly high "outliers" described in the text are represented by open circles. No relationship between NER capacity and age of donor of the human breast cells is indicated, either including all samples (unbroken line) or deleting the outlier (unbroken line). NER capacity of the PBLs significantly increases with age if all samples are included in the analysis (unbroken line), but the relationship falls just below the threshold of significance if the outlier is omitted (broken line).

these older donors. In fact, if the "outlier" is removed, the association is no longer significant ($P = 0.06$), although it remains an interesting trend. There is no evidence of a relationship between age and NER capacity in the breast samples, either when considered as combined epithelial and fibroblastic cells ($P = 0.75$), or for the epithelial ($P = 0.58$) or fibroblastic cells ($P = 0.63$) considered separately (data not shown). Removal of the breast reduction "outlier" (a different donor from that of the PBL sample mentioned above), allowed the regression to become negative, but the association remained well below the level of statistical significance ($P = 0.50$). Analysis of a possible age association was not possible for FF because all samples were harvested from infant circumcisions within the first week of neonatal life.

The S phase index, i.e., percentage of cells in the DNA synthesizing phase of the cell cycle, a measure of mitotic activity, has long been assumed to be positively correlated with DNA repair capacity [15,49]. This assumption is based on the belief that in more rapidly dividing cells there is a greater requirement for DNA repair. The S phase index can easily be derived from the autoradiographic UDS assay. As shown in Fig. 2, cells in S phase incorporate much more label than non-S phase cells, regardless of whether they have been exposed to DNA damaging agents such as UV light. Indeed, the S phase of the cell cycle is the "scheduled" DNA synthesis alluded to by the "unscheduled" DNA synthesis, or UDS used to measure NER. We have previously found that NER capacity is not correlated with the S phase index among mammalian embryonic and extraembryonic lineages [34]. In the present study, FF, breast cells and PBLs exhibited S phase indices of 25%, 20%, and 5%, respectively. We compared NER capacity to S phase index in two ways: between samples from different tissues of origin (Fig. 5A), and within the populations of PBL and breast cell samples (Fig. 5B and C). Between tissues, there is a positive correlation between the UDS results and cell proliferation, but it does not reach significance when each tissue type is represented by a single average value ($P = 0.36$). If all individual measurements are included, however, the relationship between S phase index and NER capacity is highly significant ($P < 0.001$), even when an independent cell type indicator variable is added to distinguish the three cell types (which was also significant at $P < 0.001$). This two-variable model accounted for about 40% of the variability in the NER data, suggesting that factors other than cell type and proliferative capacity influence NER capacity.

S phase indices among the population of breast reduction samples had a COV of 8% (ranging from 0.1% to 45%), and there was no evidence of a correlation with NER capacity, either including the NER outlier ($P = 0.79$), or without it ($P = 0.45$). The COV of the S phase index measurements among the population of PBL samples was much higher at 45% (range 0 to 27%), and a significant positive correlation was observed between individual S phase indices and NER capacity ($P = 0.002$). However, once again, when the outlier is removed, the association drops below the level of statistical significance ($P = 0.07$), although a trend is still evident. Age was not associated with S phase index among the breast cell samples ($P = 0.75$), but a significant association was observed among the PBL samples ($P < 0.001$)

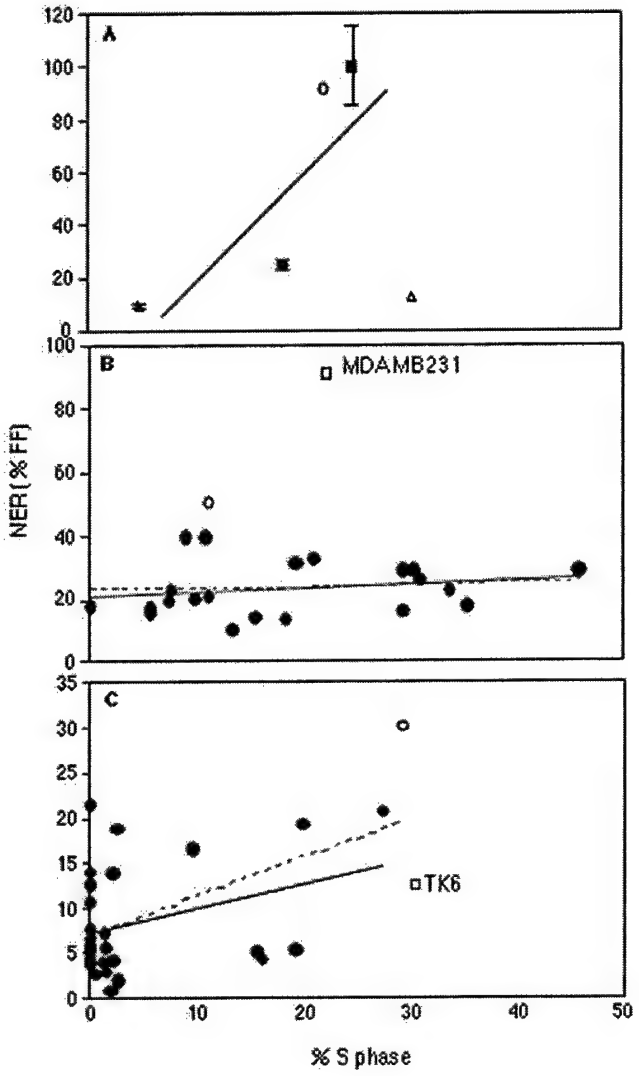

Fig. 5. S-phase index regression graphs of NER capacity for (A) the three cell types analyzed in this report, FF (filled square, ■), breast primary cultures (filled circle, ●), and PBLs (filled triangles). Error bars represent standard deviations. The relationship indicated by the line is only significant when individual points are used in the regression rather than the average results for each cell type. Also represented are data from the established breast tumor (MDA MB-231) (open circle, ○) and lymphoblastoid cell lines (TK6) (open triangle, Δ). No relationship between NER capacity and the S phase-index of human breast cells is indicated in (B), either including all samples (broken line) or deleting the outlier (unbroken line). By contrast, there is a statistically significant association between NER capacity and S phase-index in the PBL data (C), but only when the outlier sample is included (broken line). In panels B and C, the outliers are represented by open circles (○), and, for comparison, data from the appropriate established cell line from each cell type is presented (open squares, □).

Concordance with transformed cell lines

Because the UDS assay measures NER capacity relative to a control, the results are dependent on how accurately both the experimental sample and the control are quantitated. Ideally, slides are developed when the "tester" FF slides show an average of 40–50 grains per nucleus. However, with the much lower NER capacities we observed in PBLs and breast cells it was difficult to count both samples and controls with similar degrees of accuracy. With an NER capacity five to 10-fold higher than those of these samples. FF are no longer an appropriate control. An attempt to address this problem was made by measuring the NER capacities of two established cell lines with appropriate cell-type derivations, the breast tumor-derived cell line MDA MB-231 [42], and the in vitro transformed lymphoblastoid cell line TK6 [55]. MDA MB-231 was included as a second control in all experiments, and exhibited variability between experiments about on the order of the interindividual variabilities observed in the FF, breast cell, and PBL studies, with a COV of 15%. The MDA MB-231 cells exhibited an NER capacity of 91% of FF, while the TK6 line exhibited an NER capacity of 13% of FF (Fig. 1). While the NER capacity of TK6 is well within the range observed in normal lymphocytes, the NER capacity of MDA MB-231 is significantly higher than that of any of the primary breast samples, including the outlier. The unexpectedly high NER capacity of MDA-MB231 limits its usefulness as a control for breast tissue samples, while the TK6 line should be appropriate as a control for PBL studies. Our results are consistent with reports that lymphoblastoid cells have NER capacities similar to those of PHA-stimulated lymphocytes. Both of these transformed cell lines had relatively high S phase indices of 22% (MDA MB-231) and 30% (TK6). Their addition to the data examining the possible effect of S phase index on NER capacity was mixed; the MDA MB-231 data supported the previously observed trend, while the TK6 data did not (Fig. 5A). Overall, there was still no significant correlation between NER capacity and S phase index ($P = 0.58$).

Discussion

These data provide further evidence for tissue specific differences in NER capacity, suggesting that the human body is heterogeneous in its ability to deal with certain types of genotoxic insult. Because we have performed our studies on primary cultures, the results are as representative as possible of the normal physiological state of the human body. We have also characterized the variability, or "range of normal," in NER capacity for PBLs and breast cells, and to a lesser degree FF. Only one previous study has provided NER data on a human population survey, and that was performed with a transfection-based assay of transcription-coupled repair [56]. These distributions suggest that a single control "normal" sample is insufficient to provide context for experimental results. Age did not appear to play a major role in this variability. The lack of a difference in NER capacity between cell types contributing to the breast samples demonstrates that adjoining cells may exhibit similar NER activity despite their different lineage, and also suggests that cell morphology does not significantly affect the UDS assay. While NER capacity did seem to correlate with cell proliferation when different cell types were considered, there was no evidence of such an effect within either the PBL or breast cell samples. Finally, our results with the transformed lines TK6 and MDA MB-231 show that while NER capacity may be unaffected by transformation and that an established cell line may remain representative of its original tissue for this trait (TK6), other cell lines may not (MDA MB-231). These studies were possible due to our ability to grow breast cells in primary culture and due to the robust nature of the autoradiographic UDS assay.

Putative factors affecting DNA repair

In this study we saw no consistent effect of aging, cell proliferation, or cell morphology on NER capacity as measured by the UDS assay. In many types of cancer, including breast cancer, advanced age is the greatest risk factor for development of the disease. This is consistent with a proposal that DNA repair capacities decline with age, allowing greater mutagenesis and therefore carcinogenesis. Previous studies using a transfection-based assay have shown a decline in the transcription-coupled component of NER with donor age in human dermal fibroblasts, lymphocytes, and transformed lymphoblastoid cell lines [46–48]. We saw no effect of donor age among our breast cell cultures, and a significant increase in NER capacity with age of donor in our PBL samples. Rather than a loss of activity with age, as would be expected if the integrity of the NER pathway was compromised over time, this result may be associated with the known reduction in hematopoietic progenitor cells that occurs with age, increasingly compromising the diversity of the immune response [57,58]. It may be that those cells with intact NER, and by extension competence in other aspects of DNA metabolism (transcription, replication) preferentially persist into advanced age. On the other hand, our assays were performed on an uncharacterized subset of PBLs that preferentially adhered to our Matrigel-coated slides and continued to metabolize under these conditions, and we cannot know how representative these cells are of the full lymphocyte repertoire available in vivo. A recent study by Goukassian et al. [59] that examined the rate of removal of thymine dimers and 6-4 photoproducts in human dermal fibroblasts using adduct-specific antibodies reported data consistent with our study in similar age groups for breast and PBLs. It is possible that the NER associated "age effect" actually consists of a dramatic postnatal loss of activity that plateaus early in adulthood and is then maintained, or even selectively improved upon, in later years.

Comparing between tissue types, there is a suggestion of a correlation of high cell proliferation with high DNA re-

pair. More cell types will have to be evaluated to determine whether this is a generalizable trend. Addition of the cell line data did not clarify this issue, because MDA MB-231 supported the trend while TK6 did not. We found no evidence of an effect of cell proliferation on NER capacity within samples from the same tissue (PBLs and breast). These results are similar to those of a molecular analysis of expression levels of five NER genes published by Cheng et al. [49]. They found similar levels of gene expression in stimulated and unstimulated lymphocytes, but were able to distinguish the expression levels of rapidly proliferating tissues from those of slowly or nonproliferating tissues. Attempts to rationalize the endocrine theory of cancer with the somatic mutational theory have hypothesized that highly proliferating cells should have lower DNA repair capacity, due to a shortened G_1 cell cycle period [60,61], and similar arguments have been made as explanations for the observed relationship between mutation frequency and cell viability [62,63]. Our data suggest that it is doubtful that physiologically relevant changes in proliferation would have much overall impact on NER capacity.

Given our results with FF and PBLs, it was somewhat surprising that there was no observable difference in NER capacity between the two cell types (epithelial and fibroblastic) we analyzed from our breast tissue samples. This observation suggests that it is their degree of sequestration from an environmental insult, such as UV light, that might determine a cell's baseline expression of NER genes, and therefore repair activity. Alternatively, the epithelial and fibroblastic cells in our cultures may simply be different morphological manifestations of related cell types, if reversible epithelial to mesenchymal conversion takes place normally in vivo, as it does in vitro and in transformed cells [64,65]. In either case, these results suggest that differential cell morphology does not affect NER measurement by the UDS assay, either by modifying the delivery of the UV dose or the exposure of the emulsion. It is far more likely that these cells actually exhibit similar levels of NER activity than the alternative, that they manifest significantly different levels of NER that are exactly compensated by morphological effects.

Finally, a caveat: because we have demonstrated tissue specificity for NER activity, it is possible that other levels of regulation also apply to these pathway. Of concern would be cell cycle-specific regulation resulting in significant changes in NER capacity during S phase.

Tissue specificity of NER capacity and cancer risk

The importance of tissue-specificity studies can be seen in the fact that although individuals with XP have NER defects in PBLs and skin fibroblasts [66,67], they do not show heightened somatic mutation using the allele loss glycophorin A allele loss assay in their hematopoietic bone marrow cells [68]. This brings into question studies in which PBLs have been used as surrogates for many other types of tissues. Tissue specificity studies need to be performed on the normal counterparts of tissues that develop cancer. Because PBLs are relatively easily obtained, the literature is full of epidemiological studies on PBLs of people with various types of cancer, comparing the repair capacity of their PBLs with those of normal controls. It is now possible, as we have shown, to perform this work directly on the tissue(s) and cell type(s) of interest. A similar approach was taken by Monnat and co-workers [69] who performed the HPRT somatic mutation assay on human kidney epithelial cells, and found a significantly higher baseline mutation frequency than had been established in PBLs. The very low NER activity that we have observed in PBLs probably reflects their terminal differentiation and low potential for transformation.

Two previously reported epidemiological studies performed the UDS assay on PBLs from breast cancer patients [70] and from healthy women with first degree relatives with breast cancer [71]. In both of these studies, the UDS assay was performed in the presence of hydroxyurea to inhibit spontaneous DNA synthesis (S-phase cells). These studies concluded that NER capacity was reduced in the PBLs from a significantly greater number of patients and subjects with affected first degree relatives ($P < 0.01$) than in the lymphocytes of the control populations. It was suggested that subtle inherited deficiencies in NER may be a factor in breast cancer etiology [70]. In contrast, our data suggest that breast cells themselves are particularly susceptible to damage from UV-mimetic chemicals due to an intrinsically low NER activity.

The current trend in the literature appears to be away from functional assays of DNA repair and toward PCR-based molecular analyses of gene expression and of DNA repair gene polymorphism. Without knowing which of the genes are most critical to the pathway, are subject to regulation, or are functionally redundant, it is difficult to know whether molecular analysis of one or a subset of these genes will be representative of the entire NER pathway. Similarly, analyses of repair gene polymorphisms should be based on a demonstration of functional deficiency at the polymorphism itself or an associated haplotype. Serendipitous correlation with a complex biological endpoint such as cancer, may or may not be relevant.

In addition to our results demonstrating low levels of NER DNA repair in breast tissue, it has previously been shown that expression of the BER (base excision repair) glycosylases is lowest in the breast [72,73]. Intrinsically low activity of these DNA repair mechanisms in the breast might suggest an explanation for the tissue-specific cancer predisposition associated with loss of the constitutively expressed BRCA1 and BRCA2 genes, which have also been implicated in DNA repair [74]. The loss of BRCA1- or BRCA2-associated DNA repair would be expected to have the greatest impact in those cells without extensive backup capacity in this regard, such as the breast epithelium. This explanation is similar to the "redundancy" hypothesis suggested by a number of authors [75–77], but is less specific. Whereas these authors have proposed that breast, ovarian

and prostate epithelium uniquely lack redundant systems of double-strand break repair that are present in other tissues (such that loss of BRCA1/2-associated repair is less important or compensated for in most tissues but not those where cancer eventually manifests), we simply suggest that cells already at increased susceptibility to point mutations and DNA crosslinks are likely to be more affected by the increased susceptibility to double strand breaks conferred by loss of BRCA1/2 activity. This susceptibility is in contrast to cells in which compromised double strand break repair is their only vulnerability.

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Intrinsic Nucleotide Excision Repair Deficiency in Sporadic Breast Cancer

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See "Notes" following "References."

(Abstract)

Background: Genomic instability, a hallmark of carcinogenesis, can arise via loss of DNA repair capacity. We analyzed the complex, generalizable pathway of nucleotide excision repair (NER) with a functional assay in primary cultures of breast tumors. *Methods:* The autoradiographic unscheduled DNA synthesis (UDS) assay was applied to 17 unpassaged explant cultures established from stage I breast tumors. The results were compared with those obtained from 22 normal tissue explant cultures from disease-free patients undergoing breast reductions, and 1 tissue explant from the uninvolved contralateral breast of a cancer patient undergoing reconstructive surgery. Clinical parameters and possible modulating factors were considered in a multivariate analysis. *Results:* Mean tumor NER capacity was found to be 47% of that of age-matched normal controls, with 75% of the surgically confirmed stage I tumor samples lower than the lowest breast reduction value. The odds ratio (OR) for < 70% of normal DNA repair capacity = 37.4 (95% confidence interval [CI] = 17.1—82.0). No effect of patient age or tumor size, grade or hormone receptor status was found. Cell proliferation measured as the S-phase index was correlated with NER activity. *Conclusions:* Markers such as c-erbB2, epidermal growth factor receptor, cyclin D1 and c-myc have all been shown to be disregulated in only 30% of the breast tumors examined, indicating that very few molecular alterations common to the majority of breast cancers have yet been found. In striking contrast, we show that all early stage breast tumors manifest a significant deficiency of NER capacity relative to breast reduction mammoplasty cultures.

(Introduction)

Genetic instability is a hallmark of all human cancers *(1)*. It has been hypothesized that genetic instability must arise during carcinogenesis, since otherwise the multistep process would take too long to occur in a human lifetime *(2)*. The only established mechanism of genetic instability is loss of fidelity of DNA replication and/or repair *(3)*. Previous studies have focused primarily on loss of post-replicative base mismatch repair capacity *(4,5)*, as this is the genetic basis of the familial cancer predisposition syndrome, hereditary non-polyposis colorectal cancer (HNPCC) *(6-8)*. Nucleotide excision repair (NER, also known as "long patch" repair) is a complex pathway of DNA repair that can remediate any type of damage resulting in helix destabilization *(9)*. Loss of NER is the basis of the cancer prone disease xeroderma pigmentosum (XP) *(10,11)*, which is characterized by a 2000-fold increase in the incidence of UV-associated skin cancer *(12)*. In this study, we investigated the NER capacity of sporadic breast tumors relative to normal breast epithelium with the functional assay used to diagnose XP.

Recently, polymorphisms in NER genes have been studied for possible effects on skin and other types of cancers (reviewed in *13)*, and a positive association has been observed with squamous cell carcinoma of the head and neck *(14)*. Loss of NER capacity has been demonstrated in testicular cancer *(15)*, where it may have important implications for susceptibility to chemotheraputic agents *(16,17)*. Sensitivity to the clastogenicity of the tobacco smoke carcinogen benzo[a]pyrene diol epoxide, a bulky DNA adducting agent whose lesions are remediated by the NER pathway, has also been shown in breast cancer patients *(18)*.

Most studies of DNA repair and cancer have been performed either in peripheral blood lymphoctyes *(14,18)* or in established cultured cell lines *(15)*. We have developed a reliable method to culture breast epithelial cells that allows for the analysis of DNA repair directly in unpassaged primary explant cultures *(19,20)*. We believe that this system affords us a better means of investigating the somatic changes necessary for breast oncogenesis, because it allows direct analysis of the tissues of interest, rather than a surrogate (which might be affected by tissue-specific differences in DNA repair *[20,21]*). It is becoming increasingly clear that breast cancer is a heterogeneous disease by molecular and histological criteria *(22,23)*. In order to best investigate the potential role of NER in the etiology of breast cancer, we chose to restrict our study to stage I breast carcinomas. Among invasive tumors, these have undergone the least progression post-transformation, and so are most likely to exhibit characteristics of the carcinogenic process itself. Through functional analysis of such primary explants we now report a ubiquitous deficiency of NER activity in a series of sporadic stage I breast tumors, suggesting that this is an intrinsic element in the development of breast neoplasia.

Materials and Methods

Tissue samples, explant culture. Tissue from stage I invasive breast ductal carcinoma was obtained, trimmed within the tumor margin on all sides (Fig. 1). One third of each specimen was used to initiate explant culture, one-third was processed for confirmatory histological assessment, and one third was frozen for future studies. Similarly, breast reduction mammoplasty tissues were obtained from patients undergoing breast reduction surgery, and, in one case, following breast-conserving lumpectomy. All surgeries were performed at Magee-Womens Hospital, with tissue acquisition under IRB MWH-94-108. Culture conditions for all specimens, including both normal breast and tumor samples, were as previously described *(20)*, involving physical disaggregation of the tissue and culture in duplicate or more on Matrigel-coated two chamber slides in MWRIα medium. Both tumor and normal breast tissue manifested in culture generally as epithelial cells, but in some cases exhibited a fibroblastic appearance. Cells in the breast reduction mammoplasty cultures aggregated, forming "mammospheres" (Fig. 2), structures that were never formed by the tumor primary explants.

ER and PR status were determined clinically using standard peroxidase antibody staining. Five patients chose not to undergo axillary lymph node dissection to confirm negative lymph node status.

Standard inquiries pertaining to family and personal cancer histories were made at Magee-Womens Hospital at the time of diagosis. None of the subjects reported in this study had significant family histories of breast or related cancers, nor had they ever been diagnosed with another type of cancer themselves.

UDS assay. NER was measured using the autoradiographic unscheduled DNA synthesis (UDS) assay *(24)*. After seven days without passaging, actively growing primary cultures in one chamber were irradiated with 14 J/m^2 of UV at 254nm, with the second chamber shielded to act as a control. Cells were then incubated in medium containing 10 μCi/ml [^{3}H]methyl thymidine for 2 hours, the label chased with cold thymidine for 2 hours and the labeled cells fixed, dried and exposed to photographic emulsion for 10 to 14 days. As the UDS assay is a relative measure of label incorporation two positive controls were included in every experiment: fresh foreskin fibroblasts (FF) and the breast carcinoma cell line MDA MB231. After photographic development of slides the nuclei were Giemsa stained and silver grains quantitated at 1000X magnification on a Zeiss Axioskop. An average of 4.7 slides were scored for each of the stage I breast tumor samples analyzed in this study, by an average of three independent counters. An average of 165 nuclei were scored per slide, or almost 800 nuclei per sample at an average of 10.6 silver grains/nucleus. NER results from the 22 normal explant cultures have been previously reported *(20)*.

Statistical analysis. To ensure accuracy and guard against transcription errors, raw grain counts from the UDS assay were processed independently in duplicate, once using StatView (version 5.0.1, SAS Institute, Inc., Cary, NC), and once using the Data Analysis Toolpack of the Excel 2001 spreadsheet program (Microsoft Corp., Redmond, WA). The final count from slides of the same cell type within the same experiment and developed the same day were averaged together and initially expressed as a percentage of concurrently analyzed FF. The possible effects of donor age, S phase index and clinical variables such as tumor grade, size and hormone receptor status on NER capacity were

evaluated using linear regression at alpha < 0. 05. Age was evaluated both as a continuous variable and by assignment into two groups, premenopausal (< 50 years old) and peri- or postmenopausal (≥ 50 years old). Estrogen receptor (ER) and progesterone receptor (PR) status were separated into three categories, negative, positive and overexpressed. These data were also evaluated by combining the negative and overexpressed groups into one "abnormal" category. Finally, ER and PR status were also combined into a single category by assigning the higher of the two receptor status scores. Quantitative comparisons between tumor and breast reduction properties were performed using both the parametric two-tailed t-test and the nonparametric Mann-Whitney U test, both at the same level of significance. Categorical variables were also compared using the χ^2 test. In all cases, the p value reported is the highest of the three, which was always generated by the nonparametric test.

Results

Breast cancer is a biologically and clinically heterogeneous disease. In order to investigate the potential role of NER in the etiology of breast cancer, it was necessary to minimize this heterogeneity, which might reduce the power of the study. The largest subtype of breast tumors is invasive ductal or lobular carcinoma, accounting for over 80% of incident breast cancers. Within this group, stage I tumors, defined as small and non-metastatic, account for the greatest proportion of diagnosed cases, ranging from 70% in the late 90's up to the current 90%. Among invasive tumors that can progress to higher stages, these have undergone the least progression, and so should be the most informative for studying etiology. Stage I tumors, since they are so small, present a challenge to the pathologist, who would like to examine the entire tumor mass to make sure that there are not heteromorphic areas that would cause a change in the initial diagnosis. Thus, stage I tumors are less likely to be released for research purposes than larger tumors. We also fractionated the samples we obtained, freezing a portion and embedding a second for confirmatory pathological analysis (Fig. 1). Without this corroborating histopathological confirmation, samples were not included in this study. Given these considerations, practically we were only able to study tumors of 1 cm or greater, less than half of the population of stage 1 invasive carcinomas obtained at our institution. Thus, although we targeted in our investigation to the largest fraction of the most common type of breast cancer, only ~15% of newly incident cancers were appropriate for this study. Within this group, however, our rate of successful culturing for UDS analysis was 85%.

Tissue samples from 17 clinically stage I invasive breast ductal carcinomas histopathologically confirmed to be free of adjacent normal breast epithelium were processed for UDS analysis. Upon culturing, many differences were apparent between the normal and tumor samples. In our culture system normal breast epithelium on a thin coat of matrigel forms three-dimensional clusters called "mammospheres" consisting of 30-100 rounded epithelial cells (Fig. 2A). In contrast, tumor cells under the same culture conditions lack the ability to form these specific structures and, in general, assumed a more fibroblastic than epithelial appearance, despite the fact that they were derived originally from breast epithelium (Fig. 2B). The tumor cells also tended to grow as highly motile singleton cells. In addition, tumor cells in culture were confirmed using parameters used in clinical analysis of fine needle aspiration biopsies, i.e. lack of cohesiveness, enlarged nuclear to cytoplasmic ratio and the presence of prominent nucleoli.

None of our patients reported a significant family history of breast or related cancers. The sample population included two tumors of nuclear grade 1, seven of nuclear grade 2 and eight of nuclear grade 3 (Table 1). The patients ranged in age from 35 to 82 years, with six premenopausal and 11 peri- or postmenopausal. The tumors ranged in size from 1.2 to 2.0 cm. Estrogen (ER) and progesterone receptor (PR) status were categorized into three groups for each: positive, negative and overexpressed. None of these patients received pre-surgical chemo- or radiotherapy. Negative lymph node status was confirmed in 11 cases by examination of from 9 to 39 axillary lymph nodes, and in one case by examination of both the sentinel node and 4 axillary nodes. Five patients declined axillary excision, so that lymph node status could not be established. These five

patients were not significantly different from the confirmed lymph node negative population with regard to tumor size, grade, hormone receptor status or tumor S-phase index, but they were significantly older (mean of 69.6 vs. 52.5 years, $P = 0.030$). Nevertheless, all statistical comparisons reflect only the 12 patients with confirmed lymph node negative status; addition of data from the five unconfirmed lymph node patients generally yielded the same results at a higher level of confidence, reflecting the greater power of the data set from the larger population. None of these patients have recurred (median follow up of 6.5 years), providing further evidence that all non-surgically validated stage I tumors were indeed stage I.

The UDS assay provides a functional assessment of NER capacity relative to an established control. It is more relevant, however, to express the results relative to the native tissue of origin, the breast epithelium. We have previously reported UDS data from 22 samples of normal breast tissue derived from breast reduction mammoplasties *(18)*. One "outlier" was identified in this population, with an NER capacity 2.2-fold higher than that of the mean for the group. For the purposes of this report, an additional "normal" breast epithelial sample has been added to this group: from the unaffected contralateral breast of a breast cancer patient undergoing reconstructive surgery. Inclusion or exclusion of either the outlier or the sample from the cancer patient made no difference in the statistical analyses to follow. The values and probabilities reported are derived from the most conservative analysis, which excludes both the outlier and the cancer patient-derived sample.

Fig. 3 shows the NER capacity measured in breast tumor and normal primary cultures expressed as a percentage of that of the mean of the 21 conservatively chosen controls.

We have previously shown that normal breast epithelium manifests a unique level of NER activity relative to other cell types routinely analyzed with the UDS assay *(18)*. This tissue specificity was also observed in the developing mammalian embryo *(20)*.

As shown in Fig. 3, the NER capacity of the stage I tumor samples was significantly lower than that of normal breast epithelium, averaging only 47% of normal activity (P = 0.0002). Half of all tumor samples had NER capacities lower than the lowest normal epithelial sample; this proportion rose to 75% when compared to an age-matched subset of the breast reduction population. We have previously reported no effect of age on NER in normal breast (P = 0.75) *(18)*. Our control population was significantly younger (mean 35.8 years, range 19 to 65) than our cancer patients, including the subset of confirmed lymph node negative patients (P = 0.0015), since breast reduction mammoplasty is rarely performed on elderly women. Using only the 12 samples from the oldest normal donors we achieved an age match (mean 44.7 years, P = 0.17), while retaining the highly significant difference in NER capacity (P < 0.0001). Multivariate and pairwise analysis revealed no association between tumor NER capacity and patient age (P = 0.75), providing further evidence that age matching of control and patient samples is not necessary for this analysis.

Similarly, no associations were found between tumor NER capacity and tumor size, grade, ER and/or PR status. Multivariate analyses revealed an association between increased NER capacity and increased S-phase index (P = 0.013) (Fig. 4). We did not observe such an effect in the breast reduction tissues (P = 0.45) *(20)*. In the restricted data set (only tumors with confirmed negative lymph node status) compared with age-matched controls, the S-phase index of the tumors was significantly lower than breast

reduction (11.2 vs. 18.6%, $P = 0.035$); this effect was not observed in the overall data set (13.9 vs. 18.6%, $P = 0.23$). If the normal and tumor data was pooled for analysis, the association between S-phase index and NER was significant ($P = 0.025$); but did not remain so ($P = 0.14$) when a tissue identifier variable was included in the analysis (which was itself significant [$P = 0.038$]). Thus, the original observation was driven more by an intrinsic difference between the tumor and breast reduction samples rather than a shared association between NER capacity and S-phase index.

Other associations observed within this data set included an inverse correlation between patient age and tumor grade ($P = 0.015$), and a positive correlation between ER and PR status ($P = 0.043$).

Discussion

Although loss of NER has long been associated with hereditary susceptibility to skin cancer, this is the first strong evidence that it may also be involved in internal tumors. While it is well established that NER deficiency is associated with increased susceptibility to specific types of DNA damaging agents, especially UV light, it is not clear whether it is sufficient to increase the rate of spontaneous mutation. In vivo measurements of somatic mutation in blood cells of XP patients have yielded conflicting results *(25,26)*. NER is a complex pathway requiring the products of 20-25 genes *(27)*, so it represents a large target for genetic or epigenetic modification. Even if exposures are necessary to reveal mutability in NER deficient cells, due to the broad range of lesions repaired by this pathway candidate exposures include UV mimetic chemicals, DNA cross-linking agents, bulky alkylating agents and oxidative radicals *(28,29)*. Although XP patients are not generally known to manifest cancers other than in the skin and other tissues exposed to UV *(12)*, they do occur *(30)*, and their incidence may be limited by the early mortality associated with the disease *(31)*. Transgenic mice with disruption of NER genes manifest internal tumors *(32)*.

All of our stage I breast tumor samples had NER capacities below the average of normal breast reduction epithelial samples. The proportion of all stage I breast cancer tumors considered to be "deficient" in NER capacity can be estimated from our data in several ways. The most conservative method deems deficient only those tumor samples with NER values less than that of the lowest normal sample. As mentioned in the results, in the most conservative comparison, tumors whose lymph node status has been

confirmed versus age-matched controls, nine tumor samples of twelve, or 75% of the population have UDS values below that of the lowest control, and are therefore NER deficient. This drops to eight out of seventeen, or 47%, if the entire data set is used, mostly because of one low breast reduction sample (thirteen tumor samples are lower in NER than the next lowest breast reduction). Alternatively, half of all tumor samples from either set exhibit NER capacities of less than 50% of the mean of the control population, sufficient to result in an XP phenotype *(9)*. Since the only phenotype in these patients appears to be the occurrence of a breast tumor, rather than the entire set of symptoms, including birth defects, mental retardation, etc., associated with the XP syndromes, it seems overly conservative to apply such a stringent upper limit. The highest NER capacity observed in our patients with confirmed lymph node status was 0.70 of normal; one patient with unconfirmed lymph node status was higher than this (0.83) (Table 1). If 0.70 X the average of the NER capacity of the breast reduction samples is established as a cut-off, the sensitivity of detecting tumors based on reduced NER levels alone is 92%, the specificity is 77%, and the odds ratio is 37.4 (95% CI, 17.1-82.0).

Thus, at least half, and perhaps as many as all breast tumors can be considered to be deficient in NER capacity. Many molecular markers, especially activated oncogenes, have been investigated for their potential role in breast carcinogenesis and as predictive or prognostic factors, however, in contrast to our results, all but one appear to be present in one third or less of all breast tumors *(22)*. The only exception is inactivation of the DNA damage-inducible gene14-3-3 sigma *(33)*, which may be related to the effects on the NER pathway that we have observed.

A comparison of normal and tumor NER capacities from the same individuals will be necessary to differentiate between the two possible mechanisms that could be responsible for the observed tumor NER deficiency. First, breast tumors could be preferentially arising in women with constitutively low NER. This possibility is consistent with previous studies that found that DNA repair in general *(34)*, and NER in particular *(35,36)*, were lower in blood samples taken from breast cancer patients than from disease-free controls. This is not consistent with our own sample taken from the contralateral breast of a cancer patient (Fig. 3), nor of our own preliminary results in patient blood samples (data not shown), all of which are indistinguishable from normal NER capacities. The other possibility is that NER deficiency arises somatically during breast carcinogenesis. All of the known NER gene mutations are genetic recessives, and so should act like tumor suppressor genes during carcinogenesis *(9,31)*. Alternatively, mutations in genes long associated with breast cancer susceptibility, such as BRCA1, may epigenetically modulate NER activity *(37)*. It has also been shown that viral proteins inhibit NER activity *(38,39)*, suggesting that cell lines created by viral transformation are inappropriate models for measuring DNA repair and genomic instability *(40)*. Also, since most established breast cancer cell lines were derived from very late stage tumors, it is likely that they reflect genetic changes associated with tumor progression rather than oncogenesis.

Finally, our data finding a correlation between tumor NER capacity and S-phase index are inconsistent with the hypothesis that faster cell cycling should result in lower DNA repair and therefore greater susceptibility to DNA damage, one mechanistic explanation for the mitogen/mutagen theory of hormonal (and cytokine) carcinogenesis

(41,42). These data are consistent with the observation that expression of five genes in the NER pathway is elevated in proliferative vs. non-proliferative tissues *(43)* and the increase in NER observed in cultured lymphocytes when they are growth stimulated with PHA *(44)*.

The NER pathway remediates DNA damage caused by a number of genotoxic cancer chemotherapeutic drugs, including cross-linking agents such as *cis*-platinum and cyclophosphamide *(45,46)*, as well as bulky DNA adducting agents such as *N*-acetoxy-2-acetylaminofluorene (AAAF) and tamoxifen *(47,48)*. Thus, our NER data suggest that breast cancer should be particularly vulnerable to such agents. It may be useful to consider these findings in the evaluation of clinical trial data, or in the design of new chemotherapeutic regimen, as has been suggested for testicular cancer *(16,17)*. These data also support the idea that early stage tumors can be treated efficaciously by chemotherapy, a practice which has become more common over the last decade.

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Notes

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Figure Legends

Fig. 1. Schematic of the processing of dissected stage I tumor tissue sample from the post-clinical diagnosis portion of the tumor. One portion was used for in vitro culture and subsequent NER analysis, another portion was used for histopathological confirmation by creation of a second paraffin embedded block of fixed tissue and a third portion was frozen for future analysis.

Fig. 2. Primary non-diseased epithelial and tumor cultures. Photomicrographs (1000x bright field magnification) of primary epithelial cultures of **A**) normal breast reduction cells showing typical epithelial "mammosphere" architecture, and **B**) stage I breast tumor cells after processing for the UDS assay. Tumor cells manifest classical higher nuclear to cytoplasmic ratios, lack of cohesiveness and epithelial architecture and more activated looking or more prevalent nucleoli. Nuclei with dense silver grains are in S phase ("scheduled" DNA synthesis), whereas the much lighter labeling of other nuclei are due to incorporation during repair ("unscheduled" DNA) synthesis. The scale bar in A represents 4 μm.

Fig, 3. NER capacity of breast reductions vs. stage I tumors. Comparison of NER capacities of primary explant cultures established from normal breast reduction tissue and stage I ductal carcinoma. The previously described outlier in the normal population is represented by the asterisk, and the filled circle in that population represents a sample from the contralateral breast of a breast cancer patient obtained during reconstructive surgery. The filled circles in the stage I tumor population represent patients whose

negative lymph node status were confirmed surgically. Grey filled circles represent patients whose lymph node status was not confirmed.

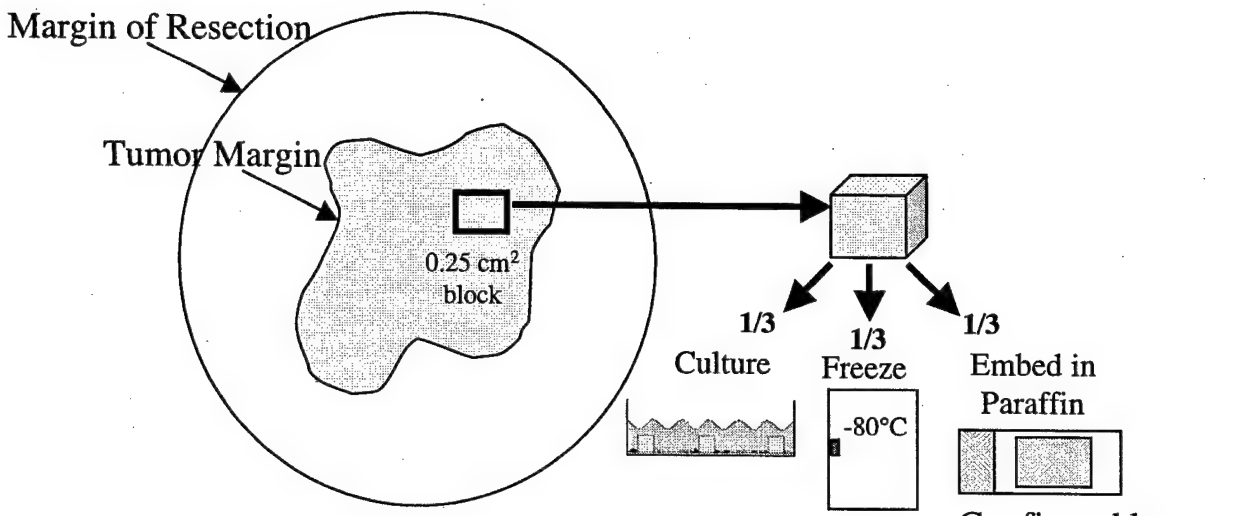
Fig. 4. Linear regression of NER capacity with S-phase index in breast tumor primary explant cultures. Filled circles represent samples from patients with confirmed negative lymph node status. Grey circles represent samples from patients with unconfirmed lymph node status. The line represents the linear regression through the combined set of samples. Exclusion of stage I tumors with unconfirmed lymph node status does not significantly affect the result ($P = 0.529$).

Table 1. Clinical and demographic characteristics of the patient population.

Table 1 Clinical and demographic characteristics of the patient population

Patient	Age (years)	Tumor Size (cm)	Tumor Grade	Tumor ER Status	Tumor PR Status	No. Axillary Lymph Nodes Examined	Tumor S-Phase Index (%)	Tumor NER Capacity[1]
10	70.6	1.2	2	positive	positive	15	0.0	0.33
19	57.3	1.3	3	negative	negative	9	6.0	0.62
25	64.8	1.5	2	positive	positive	9	10.2	0.31
27	39.2	1.6	3	negative	positive	9	15.5	0.30
35	42.2	1.7	3	overexpressed	overexpressed	19	21.9	0.60
42	34.7	1.5	3	negative	positive	39	3.2	0.32
51	63.3	1.7	2	overexpressed	negative	21	17.4	0.26
58	52.4	2.0	2	positive	positive	20	27.7	0.70
62	53.6	1.5	3	overexpressed	overexpressed	17	2.1	0.54
90	36.7	1.6	3	positive	positive	21	2.8	0.34
106	44.1	1.2	2	positive	overexpressed	10	23.7	0.59
122	70.6	2.0	2	positive	positive	1 + 4[2]	3.8	0.68
13	80.2	1.5	1	positive	negative	0	14.3	0.01
68	45.4	1.2	3	positive	positive	0	26.1	0.83
72	72.8	1.2	1	overexpressed	overexressed	0	34.7	0.50
74	68.3	1.3	3	positive	positive	0	26.9	0.42
100	81.5	1.5	2	positive	positive	0	0.2	0.49

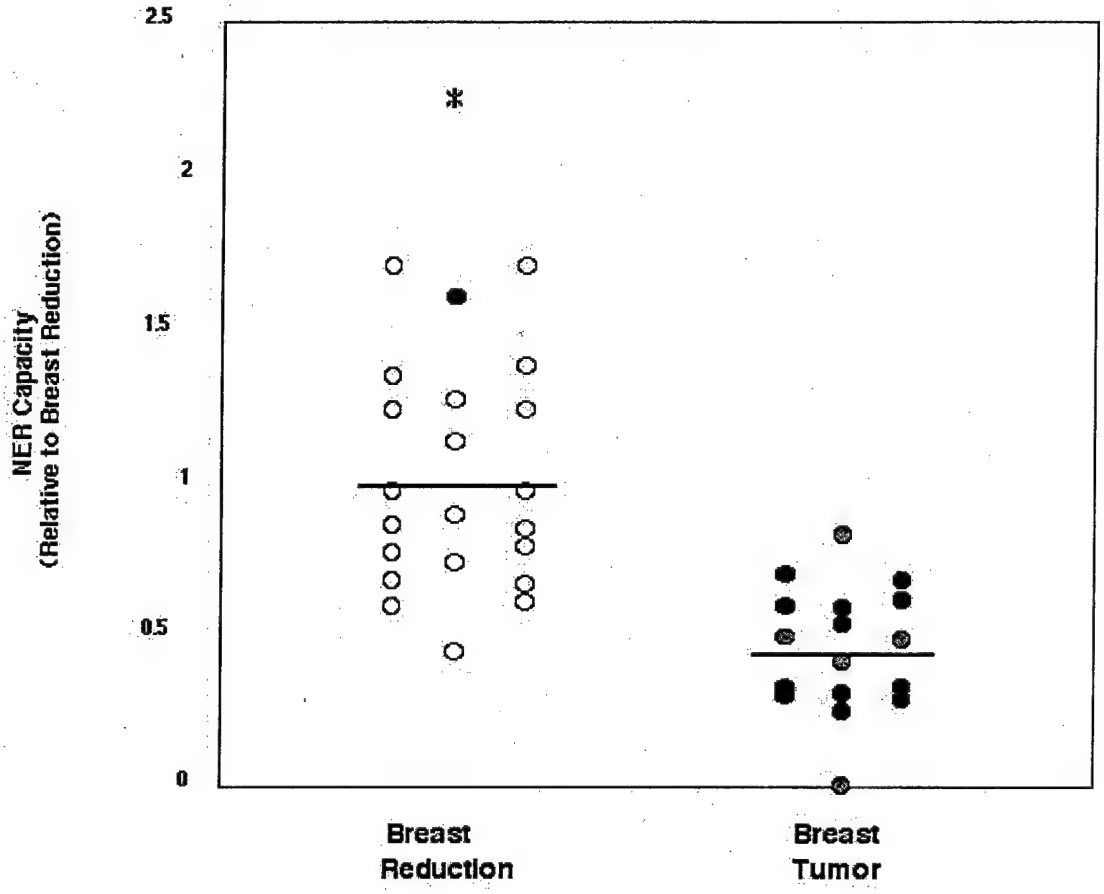
[1] relative to mean of 23 normal breast epithelial samples derived from reduction mammoplasties (20).
[2] one sentinel node and 4 axillary lymph nodes

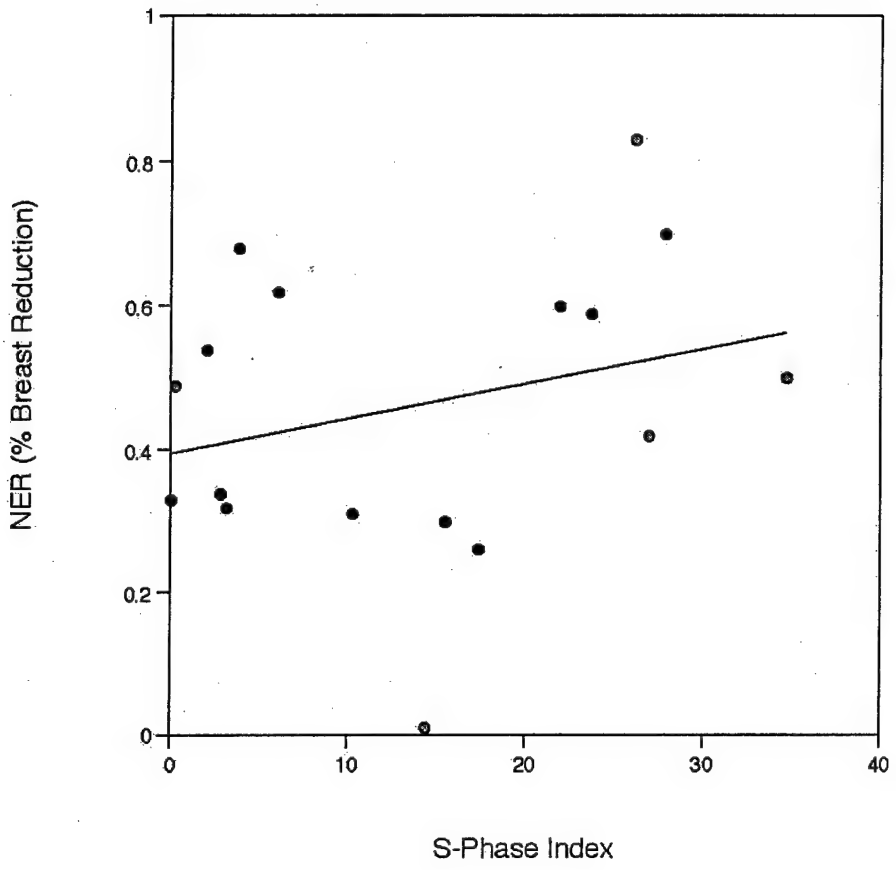


Original
Specimen
(after clinical diagnosis)

Confirmed by
pathology as invasive
carcinoma; Stage I


A
B


2.5
2
1.5
1
0.5
0
NER Capacity
(Relative to Breast Reduction)
Breast
Reduction
Breast
Tumor


NER (% Breast Reduction)
1
0.8
0.6
0.4
0.2
0
0
10
20
30
40
S-Phase Index

Normal Levels of DNA Nucleotide Excision Repair in Breast Tissue and Blood Lymphocytes of a *BRCA1* Mutation Carrier

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Abbreviated Title: Normal NER in a *BRCA1* Heterozygote

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Abstract

Genomic instability is characteristic of all cancers, including breast cancer. The underlying mechanism of genetic instability in cancer is loss of DNA repair capacity. Exposure to ionizing radiation, especially during adolescence, is a known risk factor for development of breast cancer, and increased susceptibility to DNA double strand breaks, one of the types of damage caused by ionizing radiation, is associated with loss of activity of the breast cancer-predisposing genes *BRCA1* and *BRCA2*. In sporadic breast cancer, however, there is evidence for the involvement of a different pathway of DNA repair, nucleotide excision repair (NER), which remediates small lesions that cause a distortion of the DNA helix, such as DNA cross-links and bulky adducts. To determine whether there was any association between these two types of deficient DNA repair in breast cancer, we performed the functional UDS assay for NER capacity in normal blood cells and breast tissue from a carrier of the Q1200X mutation in *BRCA1*. This patient had been newly diagnosed with a stage I breast tumor by MRI screening, and elected to undergo both a radical mastectomy of the affected breast and a prophylactic mastectomy of the contralateral breast. Using our unique culture system, we were able to grow viable explant cultures of normal breast tissue from both breasts of this patient for UDS analysis. All three samples from this patient, blood lymphocytes and normal tissue from both breasts, exhibited normal levels of NER activity when compared with our control populations. Thus, heterozygosity for inactivation of *BRCA1* does not appear to confer NER deficiency. If breast tumors from familial patients exhibit NER deficiency, however, through loss of heterozygosity of the *BRCA1* gene or some independent mechanism, then these tumors would be hypersensitive to genotoxic chemotherapeutic agents such as cyclophosphamide and *cis*-platinum, that act by generating lesions subject to the NER pathway of DNA repair.

Key words: Breast cancer, DNA repair, Nucleotide excision repair, NER, UDS, Explant culture, Individualization of chemotherapy, haploinsufficiency

Introduction

While a recent review of the properties of the tumorigenic phenotype concluded that genomic instability played only an "enabling" role in carcinogenesis[1], the facts remain that many hereditary cancer syndromes have been shown to arise from underlying defects in the fidelity of DNA replication and/or repair, and that all hereditary syndromes with DNA repair deficiencies are associated with cancer susceptibility.[2,3] This includes the hereditary breast cancer genes *BRCA1* and *BRCA2*, which have been shown to be involved in DNA double strand break repair.[4,5] DNA repair defects have also been identified in the peripheral blood cells of sporadic breast cancer patients,[6-9] but, in this case, it seems to involve another pathway of DNA repair, nucleotide excision repair (NER).[10,11] We have recently extended this observation to primary breast tumors, themselves.[12]

NER is a complex pathway of DNA repair[13] normally associated with removal of pyrimidine-pyrimidine intrastrand crosslinks ("dimers") caused by exposure to UV light. NER deficiency is the basis of hereditary xeroderma pigmentosum (XP),[14] a disease with a 1200-fold increase in incidence of skin cancer.[15] The signal for activation of the NER pathway is actually very general; any lesion causing a distortion in the DNA helix, including crosslinks caused by oxidative radicals, certain types of mismatches (purine-purine or pyrimidine-pyrimidine) and so called "bulky" adducts caused by phase I metabolism of polyaromatic hydrocarbons.[16] We have developed a method to reliably culture primary breast tissue and tumors,[17] and have used it to demonstrate that NER deficiency is characteristic of sporadic early stage breast tumors.[12] Also, it has recently been suggested that *BRCA1* expression may enhance NER activity, although this was not shown in breast cells.[18] We therefore applied the functional UDS assay for NER capacity to multiple samples of normal tissue from a patient with familial breast cancer, to

determine whether haploinsufficiency for *BRCA1* was associated with NER deficiency. If so, it would provide a mechanistic link between familial and sporadic breast cancer, since NER deficiency would predispose an individual to DNA damage from environmental chemicals such as benzo[*a*]pyrene and other polycyclic aromatic hydrocarbons present in tobacco smoke, diesel exhaust, etc.

Interestingly, we found no evidence for a constitutionally low level of NER capacity in our patient, however, suggesting more subtle defects in other genes, such as those of the NER pathway itself, are involved in breast cancer etiology. These data still impact upon patient care, however, for if the NER defect is confined to the tumor itself, chemotherapies including agents modulated by the NER pathway should be particularly efficacious in familial cancer patients.

Patient and Methods

Patient. The patient was a 36 year old woman with strong family history of breast cancer (two affected first degree relatives) recruited into a clinical trial of MRI screening for young woman at high risk for breast cancer with dense breast tissue.[19] She was subsequently found to carry a Q1200X premature termination mutation in the *BRCA1* locus. Gadolinium enhancement images revealed a small 1 cm lesion in the upper-outer quadrant of the left breast, identified pathologically as an infiltrating ductal carcinoma. The patient underwent a modified radical mastectomy of the left breast and chose to also undergo a contralateral prohylactic total mastectomy. Blood and tissue were obtained for analysis with consent under Magee-Womens Hospital/University of Pittsburgh IRB # MWH-94-108.

Control cultures. Breast reduction mammoplasty tissues were obtained from patients ages 20-70 at Magee-Womens Hospital under the above IRB. A neighboring piece of mammoplasty tissue (from the same 0.25 cm^2 sample) to that placed into primary culture was fixed and processed in paraffin. These sections were examined by a pathologist to verify the histological normality of the tissue. Breast tissue was processed as previously described.[20] Tissue was rinsed three times in PBS containing antibiotics, physically disaggregated and placed into MWRI medium[17] on a thin coat of matrigel. Peripheral blood lymphocytes (PBLs) were obtained with consent from normal healthy control subjects ages 20-50 working at Magee-Womens Hospital, Magee-Womens Research Institute or students at the University of Pittsburgh. Foreskin fibroblast (FF) tissue was obtained as discarded tissue from newborn infants after circumcision and utilized between passages 7 and 10. These control populations have been previously described in greater detail.[20,21]

Unscheduled DNA Synthesis. NER was measured by autoradiography of unscheduled DNA synthesis after UV damage (UDS).[22] After a total of 10-14 days in culture, without passaging, cultures were irradiated with UV light at 254 nm at a mean fluence of 1.2 Joules/m^2 for 12 seconds in the absence of culture medium, for a total dose of 14 J/m^2. Each sample was represented by at least two chamber slides. One chamber of each 2-chamber slide was shielded from the UV dose to be used as an unirradiated control sample. Primary cultures had not reached confluence and were still actively growing at the time the UDS assay was performed. Control FF were plated subconfluently 1-2 days before the UDS assay to insure that they also were not in a quiescent state brought on by confluence. After UV exposure, all cultures were incubated in medium supplemented with 10 μCi ml [^{3}H] methyl-thymidine (~80 Ci $mmol^{-1}$) (PerkinElmer Life Sciences, Boston, MA) for 2 hours at 37°C. Labeling medium was then replaced with unlabeled chasing medium containing 10^{-3} M non-radioactive thymidine (Sigma, St. Louis, MO) and incubated for a further 2 hours to clear radioactive label from the intracellular nucleotide pools. After incubation in the post-labeling medium, cells were fixed in 1X SSC, 33% acetic acid in ethanol, followed by 70% ethanol and finally rinsed in 4% perchloric acid overnight at 4°C. All slides were dried and subsequently dipped in photographic emulsion (Kodak type NTB2) and exposed for 10 to 14 days in complete darkness at 4°C.

The length of exposure of emulsion was determined in each experiment by preparing FF "tester" slides. After 10-12 days these tester slides were developed and grain counting was performed. If the nuclei over the foreskin fibroblasts averaged 50 or more grains per nucleus, then the rest of the experimental slides were developed. If the grain count was below this level, the remaining slides were left to expose 1-3 days longer before being developed. UDS results for the control populations have been previously reported.[21]

Grain Counting. After photographic development of emulsion, the slides were stained with Giemsa, then examined at 1000X magnification on a Zeiss Axioskop under oil emersion for grains located immediately over the nuclei of non-S phase cells. Local background grain counts were evaluated in each microscopic field, over an area the same size as a representative nucleus, and this total was subtracted from the grain count of each nucleus in that field. The average number of grains per nucleus were quantified for each side of the chamber slide, both unirradiated and irradiated. The final NER value for each slide was calculated by subtracting the unirradiated mean grains per nucleus from the irradiated mean grains per nucleus, after the initial subtraction of local background in each field. NER was initially expressed as a percentage of the activity of concurrently analyzed FF. Four FF slides were scored per experiment, by an average of three counters. There were an average of 197 nuclei per slide, for a total of almost 800 nuclei, with an average of 73.2 grains/nucleus. Six slides were scored from the ipsilateral breast tissue sample, two by each of three independent counters, and five slides were counted from the contralateral sample, again by three independent counters. An average of just over 100 nuclei were evaluated per slide for each sample, for a total of almost 600 nuclei for the ipsilateral sample and over 500 for the contralateral sample. As the NER capacities indicate, these samples had very similar counts; about 35 grains/nucleus for the ipsilateral sample and 28 grains/nucleus for the contralateral sample. Finally, six slides were evaluated for the PBL sample, two by each of three counters. An average of 195 nuclei were scored per slide (total of almost 1200), with an average of 7.5 grains/nucleus.

Statistical Analysis. To ensure accuracy and guard against transcription errors, raw grain counts from the UDS assay were processed independently in duplicate, once using StatView (version 5.0.1, SAS Institute, Inc., Cary, NC), and once using the Data Analysis Toolpack of the

Excel 2001 spreadsheet program (Microsoft Corp., Redmond, WA). The final count from slides of the same cell type within the same experiment and developed the same day were averaged together and expressed as a percentage of concurrently analyzed FF. These results were then normalized by comparison to the average for the tissue type control population (without outliers).

Results

The patient was identified as a carrier of a Q1200X truncation mutation in the *BRCA1* gene with a stage I tumor of the left breast. Blood T-lymphocytes and normal breast epithelial tissue were cultured for performance of the functional UDS assay, which requires living cells for radiolabel incorporation during repair synthesis following UV exposure. This assay is diagnostic for the inherited cancer-prone disease XP, where it is usually performed in lymphocytes or skin fibroblasts. We have developed a culture system allowing us to apply the assay in breast epithelial cells, and have demonstrated tissue-specificity in NER capacity.[21] Patient data is therefore expressed relative to the average of our tissue-specific controls.

Analysis of cultured blood lymphocytes from the patient established that they had an NER capacity of 0.996 of the average of our 33 normal samples (Figure 1). This is well above the upper limit of activity associated with hereditary XP (< 50% average normal activity) and the cut-off established in our tumor/breast reduction pooled population yielding a significant odds ratio of 37.4 (< 70% average normal activity).[17] A trend towards age dependence was noted in the analysis of the UDS data of the normal controls ($P = 0.059$);[21] addition of the patient sample supports this trend, but it still fails to reach significance ($P = 0.056$).

After surgery, pathology confirmed the presence of a small (0.7 cm diameter) malignancy in the left breast. As often happens with such small masses, no tumor sample was released to us for culture or UDS analysis (all of the stage I samples we have successfully analyzed have been 1 cm in diameter and larger[17]). We did obtain and successfully culture normal tissue from both breasts (pathologically confirmed at the time of sampling and retrospectively through examination of the tissue blocks made with our samples). Cells from these tissue samples behaved in culture very much like those from breast reduction mammoplasty of disease-free

controls, in that they grew cooperatively, clustered to form mammospheres, and went on to develop other types of three-dimensional architecture.[19,23]

The NER capacity of the ipsilateral breast epithelial sample was 1.05 times the average of our population of breast reduction epithelium (BRE) controls, clearly exhibiting no overt DNA repair deficiency (Figure 2). The contralateral sample was very similar, with an NER capacity of 1.17 times BRE normal (Figure 2). Although the NER values of these two samples from the same patient are similar, they are not close enough to distinguish themselves as coming from the same individual ($P = 0.16$) Our earlier analysis of NER in our normal population revealed no effects of age or cell proliferation (as represented by the S-phase index), and these patient samples are consistent with those results.

Discussion

We have shown that all sporadic stage I breast tumors are deficient in NER capacity relative to normal breast epithelium.[12] Moreover, normal adjacent tissue from these patients also manifest NER deficiency in 75% of cases.[24] These results suggest that most sporadic breast cancers occur in women with constitutively low levels of NER, consistent with measures of DNA repair capacity in their blood lymphocytes.[10,11] Since the established breast cancer predisposing genes *BRCA1* and *BRCA2* are involved in DNA repair,[4,5] it is possible that they are responsible for, or contribute to this effect in the sporadic breast cancer population. Our results from a single patient indicate, however, that while overexpression of *BRCA1* may enhance NER,[18] haploinsufficiency for this gene does not necessarily result in an NER deficiency.

NER deficiency is most often associated with XP, sensitivity to UV-induced DNA damage and skin cancer.[15] The NER deficiency of XP patients is manifested in other tissues, however, as shown by their high spontaneous frequency of mutation in blood lymphocytes[25] and the occurrence of other types of tumors.[26] Indeed, survivors of sporadic skin cancer have been shown to be at increased risk of other cancers, including tumors of the lung, breast, colon and head and neck.[27,28] Sporadic skin cancer patients also manifest an NER defect in blood lymphocytes,[29,30] as do patients with lung[31] and head and neck[32] cancer. Polymorphisms in NER genes have been associated with increased risk of skin cancer[33], oligoastrocytoma[34], squamous cell carcinoma of the head and neck[35], and lung cancer.[36,37] Hypersensitivity to clastogenesis from UV light, 4-nitroquinoline-1-oxide (4NQO) or benzo[*a*]pyrene diol epoxide (BPDE), all of which cause DNA damage remediated by the NER pathway, has been reported in blood cells from skin cancer patients[38], as well as lung and head and neck cancer patients[39,40] (this hypersensitivity has been associated with reduced NER capacity[38]). Loss of heterozygosity of

NER genes has been demonstrated in oral squamous cell carcinoma[41], and reduced NER capacity has been shown in testicular cancer cell lines (although this deficiency was not demonstrated against tissue-type controls).[42] All of these data suggest that inherent NER deficiency and/or somatic loss of NER is involved in the etiology of many types of cancer. Consistent with our results suggesting a constitutive, and therefore hereditable deficiency, are studies showing deficient DNA repair,[43] and specifically NER capacity[44] in blood samples from relatives of sporadic breast cancer patients.

The possibility that *BRCA1* heterozygotes manifest a cellular phenotype promoting carcinogenesis has been investigated with conflicting results; most studies show no hypermutability in these cells or individuals,[45,46] while others report that they have high spontaneous frequencies of mutation[47] and are hyperinducible.[48] We have found that the mutation frequency in *BRCA1* heterozygotes is significantly elevated in young patients, but that it does not further increase with age, such that there is no significant difference in the older population.[49] A similar profile of age-dependent mutagenesis has been reported for patients with Down syndrome.[50]

Haploinsufficiency of BRCA1 has been reported to arise somatically in sporadic breast tumors, either by gene deletion or epigenetic inactivation via promotor methylation.[51] *BRCA1* expression has been found correlate with response to anthracycline-based chemotherapy in model systems,[52] familial[53] and sporadic[54] breast cancer patients. These finding suggest that BRCA1 haploinsufficiency has a clinical correlate which predicts response to chemotherapy. Anthracyclines are topoisomerase II inhibitors, creating single stranded nicks in DNA, but their efficacy has also been shown to be modified by base excision repair pathways,[55,56] although not

specifically NER. Anthracyclines[57] and PAHs[58] have been found to inhibit expression of *BRCA1*.

The relative NER capacities of tumor and normal tissue have important practical implications. Constitutive or early occurring NER deficiency confers sensitivity to certain types of carcinogenic agents, such as tobacco smoke metabolites. On the other hand, modulations in NER capacity that are confined to the tumor have important connotations for treatment. NER deficiency, such as has been seen in testicular cancer[42], suggests sensitivity to a range of chemotherapeutic drugs, including alkylating agents (cyclosphosphamide), cross-linking agents (cis-platinum) and bulky DNA adducting agents (melphalan). Individualization of chemotherapy based on some aspect of NER expression is being pursued in colon,[59] as well as testicular cancer.[60,61] Alternatively, overexpression of NER is a known mechanism of drug resistance,[62,63] and chemical modulation of NER capacity is being investigated as a means of enhancing *cis*-platinum efficacy in advanced ovarian cancer.[64] If breast tumors from familial patients exhibit NER deficiency, while their normal tissues exhibit normal levels of this type of DNA repair, then the tumors would be hypersensitive to the genotoxic agents mentioned above, and more efficacious treatments may be developed based on the increased difference in sensitivities exhibited by normal and malignant tissue.

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Figure Legends

Figure 1. Comparison of the NER capacity of a PBL sample from our BRCA1 mutation carrier patient with those of a population of disease-free controls. The dark horizontal line indicates the average for the normal population, while the dotted lines indicate upper limits for residual NER activity in patients with the hereditary NER deficiency disease XP (0.50) and the cut-off established in our breast tissue study that identified tumors with high sensitivity and specificity (0.70).

Figure 2. Comparison of the NER capacities of a two samples of normal breast epithelium from our BRCA1 mutation carrier patient with those of a population of disease-free controls who underwent breast reduction mammoplasty. The dark horizontal line indicates the average for the normal population of breast reduction epithelium (BRE), while the dotted lines indicate upper limits for residual NER activity in patients with the hereditary NER deficiency disease XP (0.50) and the cut-off established in our breast tissue study that identified tumors with high sensitivity and specificity (0.70). The patient sample on the left was derived from the ipsilateral (left) breast, while the sample on the right was from the contralateral (right) breast.

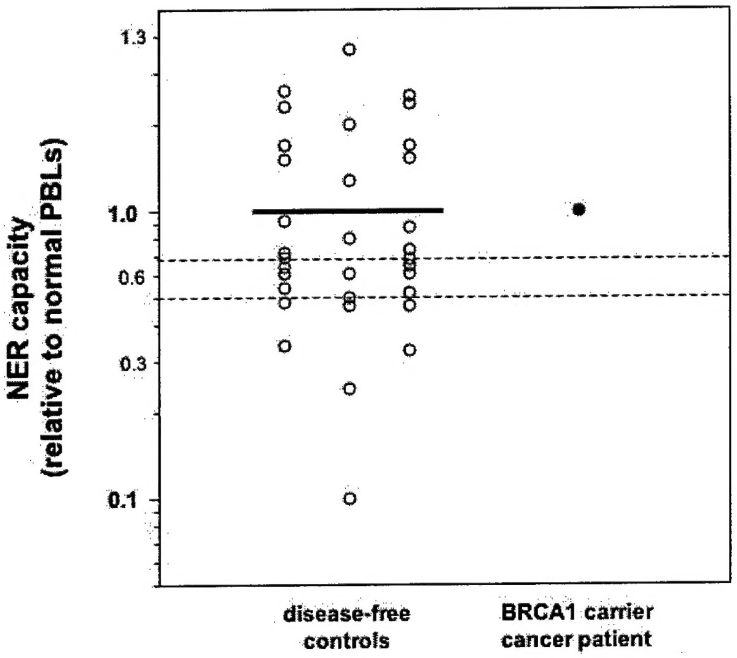
NER capacity
(relative to normal PBLs)
1.3
1.0
0.6
0.3
0.1
disease-free
controls
BRCA1 carrier
cancer patient

Figure 1.

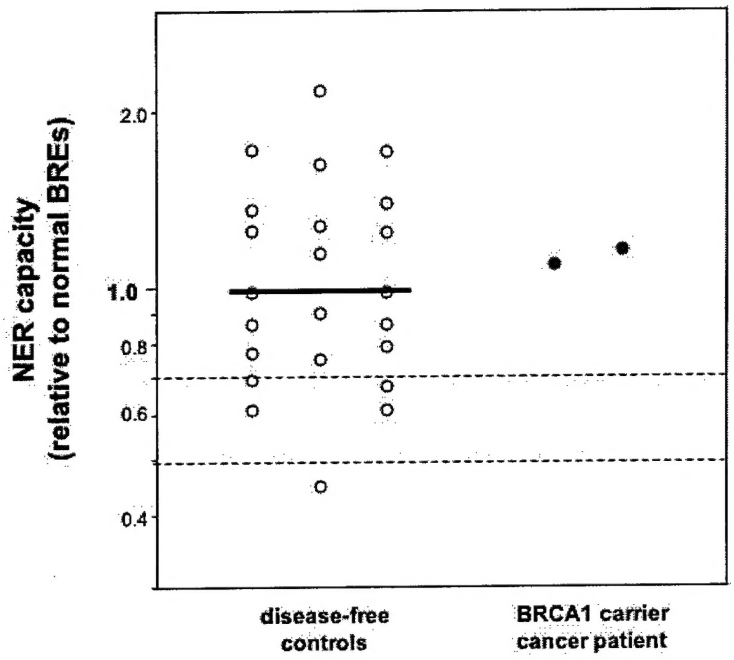
NER capacity
(relative to normal BREs)
2.0
1.0
0.8
0.6
0.4
disease-free
controls
BRCA1 carrier
cancer patient

Figure 2.